Journal of Biological Regulators and Homeostatic Agents
Volume 29, No. 4 (Supplement 1) October-December, 2015

PTEN ELEVATION, AUTOPHAGY AND METABOLIC REPROGRAMMING MAY BE INDUCED IN HUMAN CHONDROCYTES DURING STEROIDS OR NUTRIENT DEPLETION AND OSTEOARTHRITIS

O. GALASSO[1], S. PANZA[2], M. SANTORO[3,4], M.B. GOLDRING[6,7], S. AQUILA[4,5] and G.GASPARINI[1]

[1]Department of Orthopaedic Surgery, Magna Graecia University, Catanzaro, Italy; [2]Department of DiBEST, [3]Post-graduate School Clinical Pathology, [4]Centro Sanitario, [5]Department of Pharmacy and Sciences of Health and Nutrition, University of Calabria, Rende, Italy; [6]Engineering Repair and Regeneration Program, Hospital for Special Surgery, New York, NY, USA; [7]Department of Cell and Developmental Biology, Weill Cornell Medical College, New York, NY, USA

S. Aquila and G. Gasparini are joint senior authors.

The exact mechanisms controlling the development and progression of osteoarthritis have not yet been clarified. Our aim was to investigate new pathomechanisms, with an emphasis on novel molecular targets that might regulate human chondrocytes in osteoarthritis. As a model for studying cell survival and metabolism, C-28/I2 and T/C-28a4 human chondrocytes were grown in complete medium, in dextran-coated charcoal treated medium and in serum-free medium. Healthy and osteoarthritic human cartilage samples were obtained from discarded surgical material. Cell survival, PTEN, AKT, Beclin1, AMBRA, AMPK and glucose/triglyceride metabolism were evaluated by immunoblotting and spectrophotometric assays. Starvation and steroids depletion decreased cell survival concomitantly with PTEN elevation, repression of the PI3K/AKT signaling axis and autophagy activation. These experimental conditions promoted the accumulation of glucose, decreased levels of G6PDH and resulted in differential expression of OXPHOS complexes. Furthermore, they induced the expression of AMPK, reduced triglyceride levels and increased lipase activity, which was accompanied by a change in chondrocytes toward a fibroblast-like morphology. In osteoarthritic human cartilage, increased PTEN, AMPK and autophagy reflected the chondrocyte responses observed during starvation and steroids depletion. In conclusion, we defined the metabolic phenotype of human chondrocytes, in which both starvation and steroids depletion induce the activation of PTEN, AMPK and autophagy signaling, concomitant with metabolic reprogramming. Our data may aid in the development of novel in vitro models for the discovery and design of drugs or nutraceuticals capable of ameliorating the course of osteoarthritis.

Osteoarthritis (OA) is a major cause of morbidity and disability and the burden of this disease has been steadily gaining importance in recent decades with the aging of the world's population (1). Although important advances have been made in understanding the pathophysiological processes of OA, the exact

Key words: osteoarthritis, chondrocyte cell lines, starvation, steroids, autophagy, cell death, glucose metabolism, lipid metabolism

Mailing Address: Saveria Aquila, MD,
Associate Professor of Human Anatomy,
Department of Pharmacy and Sciences of Health and Nutrition,
University of Calabria, Via Pietro Bucci, Cubo 34B,
Arcavacata di Rende, Cosenza, Italy
Tel.: +39 3389078488 Fax: +39 09613647104
e-mail: saveria.aquila@libero.it

0393-974X (2015)

mechanisms that could be targeted to control the development and progression of this disease have yet to be determined. Because it is difficult to reverse the process once cartilage is eroded, there is great interest in strategies that prevent damage at early stages or maintain homeostasis.

Apoptotic chondrocytes have been detected during endochondral ossification (2), in hyaline cartilage during aging (3), in rheumatoid arthritis (4) and in OA (5). In OA, increased chondrocyte apoptosis has been correlated with the severity of cartilage damage (6). Various lines of evidence suggest that apoptosis is the main type of death in chondrocytes and that serum starvation of these cells induces apoptosis (7). Phosphatidylinositol-3 kinase (PI3K) and its downstream target serine-threonine kinase (AKT) are classic cell survival signals that have an important role in regulating bone development (8). The PI3K/AKT pathway is also related to autophagy, a physiological process involved in the degradation and turnover of cytoplasmic organelles. It has recently been claimed that autophagy is a protective mechanism in normal cartilage and that its aging-related loss is linked to cell death and OA (9). A key negative regulator of the PI3K/AKT pathway is the tumor suppressor gene phosphatase and tensin homolog deleted on chromosome ten (PTEN) (10). However, the role that PTEN plays in cartilage degeneration during OA has not been completely elucidated. The survival pathways that function in the regulation of cellular metabolism have received intense study.

Studies suggest that in osteoarthritic cartilage damage occurs simultaneously with metabolic dysfunction and nutrient imbalance (11, 12). Several proteins involved in lipid metabolism are significantly downregulated in osteoarthritic chondrocytes, suggesting that impaired lipid metabolism may be a critical factor in OA (13). Recently, a novel area of cartilage nutraceutical research investigated glucose metabolism and signaling pathways as suitable targets for modulation of the behavior and biosynthetic activity of articular chondrocytes (14). However, detailed molecular studies evaluating the metabolic profile of chondrocytes are limited.

Cell lines such as C-28/I2 and T/C-28a4 have been generated as a reproducible source of human chondrocytes (15) and are commonly used as models for studying cartilage breakdown and repair and for evaluating chondrocyte function. In the present study, we decided to use these cell lines to develop a model to examine the metabolic phenotype of human chondrocytes to better define the molecular mechanisms governing their viability and metabolism. To accomplish this, the cell lines were grown in complete medium (MC) and then transferred to medium containing serum treated with dextran-coated charcoal (DCC), to strip steroids from serum, or to serum-free (-S) medium. The action of steroids on cartilage is hardly being elucidated and the clinical impact of these hormones in articular cartilage is completely unclear to date. To determine whether our findings reflected clinical outcomes, human healthy and OA cartilage samples were also examined.

MATERIALS AND METHODS

Chemicals

Acrylamide bisacrylamide (A2917) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Monoclonal mouse antibodies (Abs) against human Beclin1 (Cell Signaling technology, Milan, Italy, #3738), activating molecule in Beclin1-regulated autophagy (AMBRA) (N-18), b-actin (AC-15), PTEN and AMP-activated protein kinase (AMPK) and polyclonal rabbit Abs to AKT (H-136) (Santa Cruz Biotechnology, Milan, Italy) were used in this study. Type II collagen was probed with polyclonal antibodies raised against amino acids 873-1072 of Collagen a1 Type II of human origin, which map near the C-terminus (Santa Cruz Biotechnology, Milan, Italy).

Cell cultures

Immortalized C-28/I2 and T/C-28a4 human chondrocytes were evaluated in the current study, which are widely used to study chondrocyte growth and differentiation (16). The cells were plated and grown for 12, 24, or 48 h in complete medium (MC) consisting of DMEM/F12 with or without phenol red (Sigma, Milan, Italy) plus 10% fetal bovine serum (FBS, Atlanta Biologicals, Milan, Italy), 1% penicillin/streptomycin (Cellgro, Milan, Italy) and 200 mM glutamine (Thermo Scientific, Hyclone, Milan, Italy) at 37°C in a humidified cell culture incubator with 5% carbon dioxide. Other sets of experiments included cells grown in MC and then starved for 12, 24, or 48 h in medium containing serum treated with dextran-coated charcoal (DCC) to strip the steroids from the serum or in serum-free medium (-S). Monolayer-cultured chondrocytes between passages 1

and 7 were used.

Human tissues

Human articular cartilage was obtained with institutional review board approval from the discarded surgical material of patients who underwent surgery for total joint replacement in the Department of Orthopedics of the Magna Graecia University of Catanzaro, Italy.

Approval was obtained by the local ethics committee and the patients had given their informed consent to the project prior to their operation. In total, 14 donors suffering from OA were recruited between January 2010 and January 2012 (6 women and 8 men, with a mean age of 67.9±8.1 years and a body mass index of 28.3±2.5). The diagnoses were based on established clinical, biochemical and radiological criteria (17, 18).

Specifically, 7 of the 14 individuals had knee OA and cartilage was taken from femoral condyles and the tibial plateau. The remaining donors (i.e., 7 patients) suffered from hip OA and the cartilage was harvested from whole femoral heads. Radiological classification of each OA joint was carried out according to the Kellgren-Lawrence (19) scales by two independent radiologists. Cohen's kappa coefficients for inter-observer and intra-observer reliability in scoring were 0.80 and 0.84, respectively. A consensus decision on the scores was reached in a final common readout. A III/IV grade OA was noted in 11 patients and a I/II grade OA was noted in 3 patients.

Patients with secondary OA or those who had previously received intra-articular injections of corticosteroids, hyaluronic acid, or non-steroidal anti-inflammatory drugs were excluded from the study. Two patients without OA or other diseases that could potentially alter the articular cartilage served as controls. Specimens were excised from the superficial and deep layers of the articular cartilage using disposable scalpel blades, avoiding the calcified layer and the subchondral bone. Approximately 0.5 cm^3 of cartilage was dissected from all of the specimens from the patients. After the cartilage was removed from the joint, it was extensively washed with an isotonic saline solution to remove residual blood or synovial fluid. The samples were immediately immersed in liquid nitrogen and stored at -80°C until analysis.

Cell survival analysis

Cells (3×10^4 cells/mL) were plated in 24-well plates and grown in the different experimental conditions. The MTT assay was performed as follows: one hundred microliters of 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium (MTT) (2 mg/mL) (Sigma Aldrich, Milan, Italy) was added to each well and the plates were incubated for 2 h at 37°C, followed by medium removal and solubilization in 500 mL DMSO. The absorbance was measured with an Ultrospec 2100 Prospectrophotometer (Amersham-Biosciences, Milan, Italy) at a wavelength of 570 nm. The results are expressed as a percentage of the controls, as determined by standardizing the cells in MC to 100%.

Immunoblotting

Western blotting was performed as previously described (20). Frozen human articular cartilage samples were ground with a mortar and pestle under liquid nitrogen, added to RIPA buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate with a protease inhibitor cocktail) and homogenized on ice with an Ultra-Turrax T25 homogenizer (Janke & Kunkel, Staufen i. Br., Germany). After centrifugation for 30 min at 16,000×g, the supernatant was transferred to Eppendorf tubes. The concentration of protein was determined using a protein assay kit (Bio-Rad, Milan, Italy, 500-0006) at 595 nm.

Glucose assay

The glucose assay used the glucose oxidase-peroxide reaction. Glucose was oxidized to δ-gluconolactone with the concomitant reduction of the flavin adenine dinucleotide (FAD)-dependent enzyme glucose oxidase. The reduced form of glucose oxidase was regenerated to its oxidized form by molecular oxygen to produce hydrogen peroxide. Finally, with horseradish peroxidase as a catalyst, hydrogen peroxide was reacted with 3.5-dichloro-2-hydroxybenzenesulfonic acid and 4-aminoantipyrine to generate a pink dye with an optimal absorption at 514 nm.

G6PDH activity assay

The conversion of $NADP^+$ to NADPH, catalyzed by G6PDH was measured by the increase in absorbance at 340 nm, as previously described (21, 22).

Triglyceride assay

Triglyceride levels were measured in cell lysates in duplicate using a glycerol-3-phosphate oxidase (GPO) and peroxidase (POD) enzymatic colorimetric method according to the manufacturer's instructions, as previously described (23, 24). The data are presented as mg/10^6 cells.

Lipase activity assay

Lipase activity was evaluated by the method of Panteghini et al. (25), which is based on the use of 1.2-o-dilauryl-rac-glycero-3-glutaric acid-(6'-methylresorufin) ester (DGGR) as the substrate, as previously described (22).

Statistical analysis

Statistical analysis was performed using an ANOVA

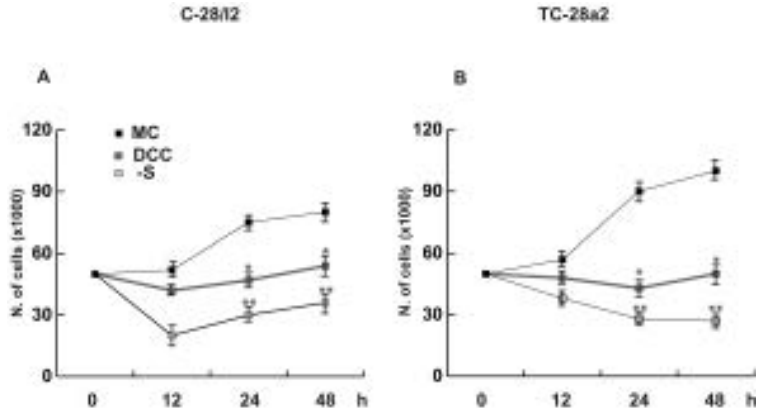


Fig. 1. *Time-course analysis of viability in C-28/I2 (**A**) and T/C-28a4 (**B**) chondrocyte cell lines. The cells were cultured in complete medium (MC). After three passages, one set of experimental cells included cells grown in MC and the other sets of cells were starved in medium with serum treated with dextran-coated treated charcoal (DCC) or in serum-free medium (-S). All three experimental conditions were analyzed at 12, 24 and 48 hours (h). The squares in the graphs represent the mean ± SEM of six independent experiments performed in triplicate. ■: MC; ▩: DCC; □: -S; *:P<0.05; **;P<0.02 versus MC cells.*

followed by Newman-Keuls testing to determine differences in the means. $p<0.05$ was considered statistically significant. All statistical analyses were performed using SPSS software (v. 17).

RESULTS

Starvation decreases cell survival in chondrocytic cell lines, C-28/I2 and T/C-28a4

The C-28/I2 and T/C-28a4 cells were exposed to MC, DCC or -S medium and cell survival was evaluated by the trypan blue exclusion assay. As shown in Fig. 1, in both cell lines, viability decreased significantly in DCC and -S medium compared with the cells in MC. The survival of the -S cells was lower compared with those incubated in DCC medium. Additionally, it appeared that the survival of T/C-28a4 cells was higher than that of the C-28/I2 cells in MC.

PTEN *elevation and autophagy were induced in C-28/I2 and T/C-28a4 cells upon starvation*

PTEN is expressed in chondrocytes (26) and a tight relation between PTEN and autophagy has been reported (27). We therefore examined PTEN and AKT by immunoblot analysis in C-28/I2 and T/C-28a4 cells incubated in MC, DCC, or -S medium. PTEN was increased in cells cultured in DCC or -S medium but not in a time-dependent fashion. Consistently, AKT expression was almost completely abolished in the DCC and -S incubation media. The results were similar for both cell lines (Fig. 2A).

In other differentiated cell types, starvation induces autophagy. In our study, Beclin1 and AMBRA, important autophagy markers, increased under the DCC and -S experimental conditions (Fig. 2B). Collectively, these data suggest that nutrient depletion induces autophagy in human chondrocytes in a manner similar to that in various differentiated cells.

Starvation affects glucose metabolism in C-28/I2 and T/C-28a4 cells

In the cellular model we used, the metabolic phenotype has never been investigated. We first analyzed glucose utilization in these cells by evaluating glucose content and G6PDH activity. Starvation induced the accumulation of glucose in both cell types, although higher levels were observed in the T/C-28a4 cells (approximately 87% in the T/C-28a4 cells and approximately 48% in the C-28/I2 cells) (Fig. 3A, B). Chondrocyte starvation resulted in a strong and significant reduction in G6PDH activity, the first enzyme of the pentose

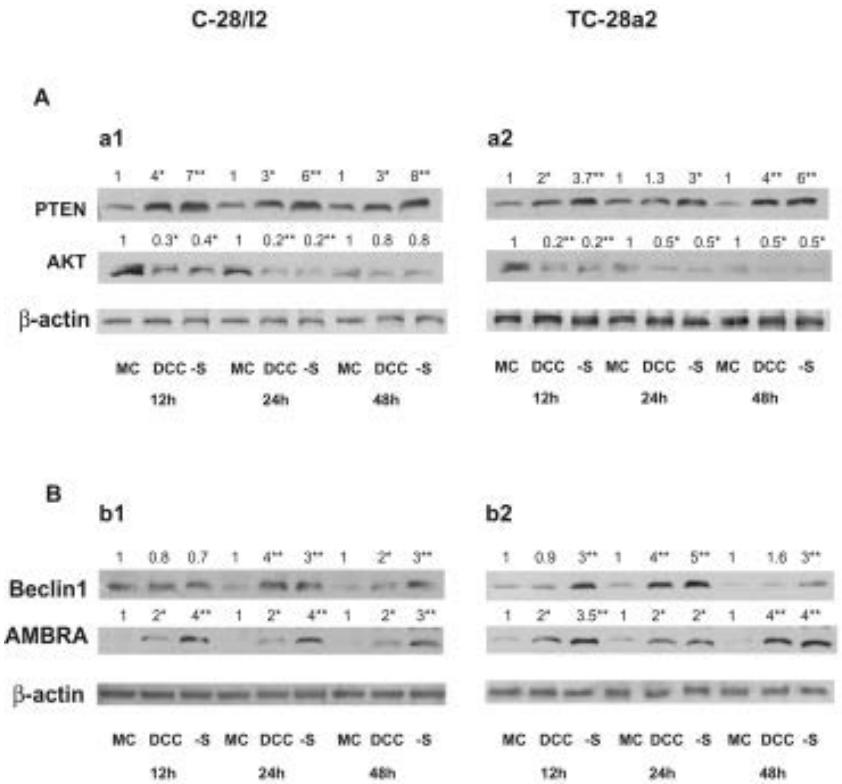


Fig. 2. *Starvation induces PTEN elevation and autophagy in C-28/I2 and T/C-28a4 cells.* ***A****): Western blot analysis showing PTEN and AKT at 12, 24 and 48 hours in C-28/I2 (panel* ***a1****), T/C-28a4 (panel* ***a2****), cells grown in MC, DCC, or -S medium;* ***B****): Western blot analysis showing Beclin1 and AMBRA at 12, 24 and 48 h in C-28/I2 (panel* ***b1****) and T/C-28a4 (panel* ***b2****) cells cultured under the experimental conditions mentioned above.* β-actin was used as a loading control. *The autoradiographs show the results of one representative experiment and the numbers on the top of the blots are the means of three independent experiments in which band intensities were evaluated in terms of arbitrary units of optical density and expressed as fold over control (MC), which was assumed to be 1. *P<0.02 and **P<0.01: versus MC-treated cells at each time considered.*

phosphate pathway (PPP) (Fig. 3C, D), which matched the increased glucose content observed in the cells cultured in DCC or -S medium.

Starvation alters the bioenergetic profile of C-28/I2 and T/C-28a4 cells

Normal cells primarily employ mitochondrial oxidative phosphorylation to generate the energy needed for cellular processes. However, in different settings cells may display aberrant bioenergetic phenotypes that range from highly glycolytic to partial or enhanced mitochondrial oxidative phosphorylation (OXPHOS). In this regard we investigated the effect of starvation on the bioenergetic balance in the cell lines by analyzing the expression of OXPHOS components and AMPK. As shown in Fig. 4A, in C-28/I2 cells, starvation increased the levels of complex II (CII) and complex V (CV) of the respiratory chain at 24 h, whereas the amount of complex I (CI) was reduced. Expression of CII and CV was reduced after 48 h of starvation. The T/C-28a4 cells did not show a similar response pattern, although a reduction in CI was noted at 48 hours (Fig. 4B). Interestingly, in both cell lines, the near absence of complex IV (CIV) was observed. In addition, after longer exposure than that presented in Fig. 4A and B, weak bands at 16 kDa were obtained.

AMPK is a crucial regulator of energy metabolism at the cellular level (28, 29) and its activity is closely linked to ATP production. Our

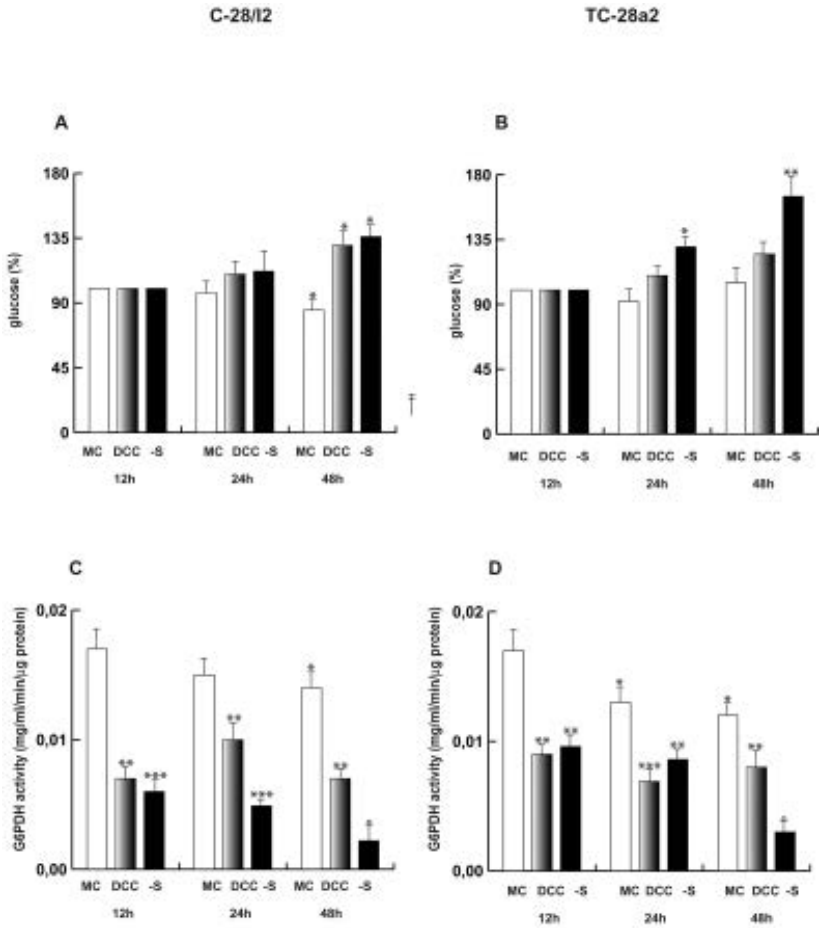


Fig. 3. *Starvation influences glucose metabolism in human chondrocytes cell lines.* ***A, B****): Glucose content was evaluated in C-28/I2 (****A****) and T/C-28a4 (****B****) cells at 12, 24 and 48 h and in MC, DCC, or -S medium. The results are presented as the percentages of glucose content with respect to the 12-hour time point;* ***C, D****): G6PDH activity in C-28/I2 (****C****) and T/C-28a4 (****D****) cells at 12, 24 and 48 h and in MC, DCC, or -S medium. The columns represent the mean ± SEM of six independent experiments. *P<0.05, **P=0.02, ***P<0.01 and* °P*<0.001 versus MC-treated cells at 12 h.*

data showed that AMPK is greatly increased upon starvation, which is consistent with both the absence of CIV and the reduction in CI observed in the two cell lines (Fig. 4C, D). Together, these data suggest that slow or delayed bioenergetic metabolism exists in chondrocyte cell lines, particularly upon steroids deprivation or starvation.

Starvation promotes the reduction of lipids in C-28/I2 and T/C-28a4 cells

To provide an almost complete view of the metabolic phenotype of human chondrocytes we evaluated triglyceride content and lipase activity. As shown in Fig. 5A and B, the DCC or -S cells exhibited reduced triglyceride levels compared with the cells incubated in MC, showing a slight time-dependent decrease in the T/C-28a4 cells. These results are in agreement with the increased time-dependent lipase activity observed in both cell lines (Fig. 5C, D). However, the lipase activity of the cells incubated in MC decreased in a time-dependent manner. Thus, our data indicate that steroids or nutrient depletion produces an altered metabolic phenotype in the C-28/I2 and T/C-28a4 cell lines.

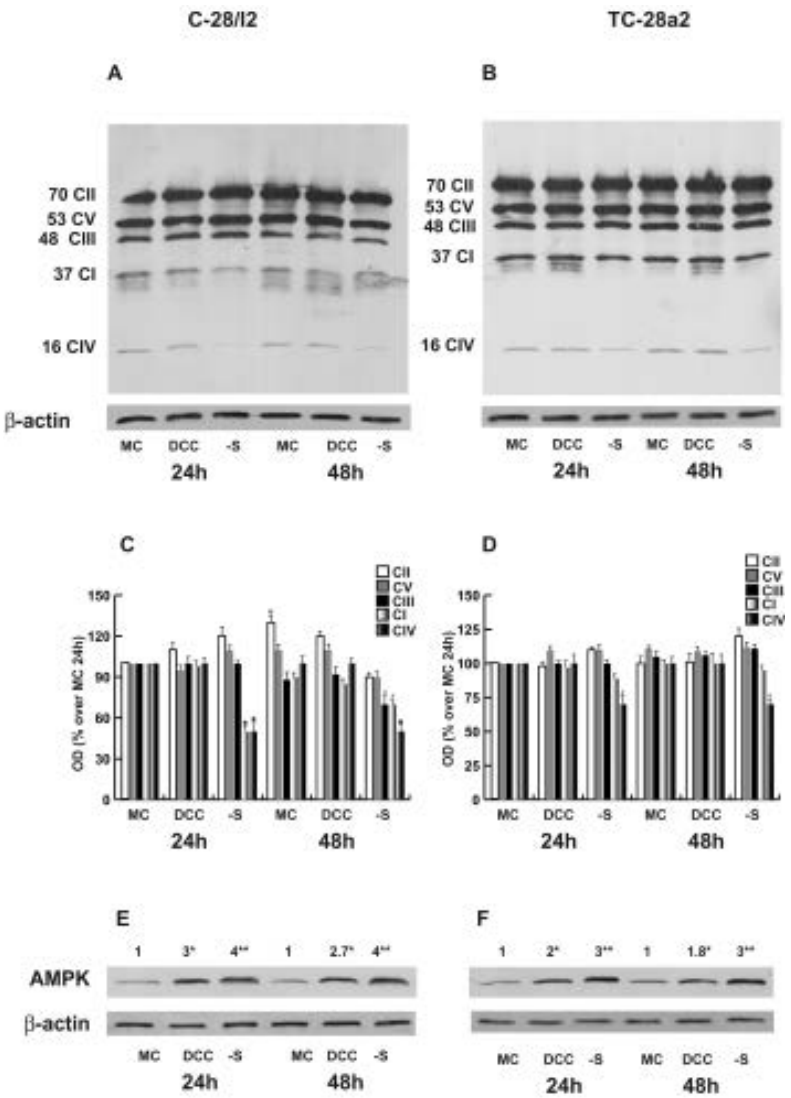


Fig. 4. *Starvation alters the bioenergetic profile of C-28/I2 and T/C-28a4 cells.* ***A, B****): Immunoblot analysis of the OXPHOS components;* ***C, D****): Quantitative representation after densitometry of the OXPHOS components; the columns are mean of three independent experiments in which band intensities were evaluated in terms of arbitrary units of optical density and expressed as a % with respect to the control (MC at 24 h), which was assumed to be 100 %. *P=0.05, #P<0.05 and •P<0.01 versus MC-treated cells at 24 h.* ***E, F****): AMPK in C-28/I2 and T/C-28a4 cells, respectively. The experimental conditions are indicated in the figure.* β-actin was used as a loading control. *The autoradiographs show the results of one representative experiment repeated at least three times. The numbers on the top of the blots are the means of three independent experiments in which band intensities were evaluated in terms of arbitrary units of optical density and expressed as fold over control (MC), which was assumed to be 1. *P<0.05 and **P<0.01 versus MC cells at each time considered.*

Nutrient or steroids depletion induces a switch from the chondrocyte morphological phenotype to a fibroblast-like phenotype

As both PTEN and AMPK regulate cell metabolism, function and differentiation, the increased amounts of PTEN and AMPK upon cell starvation may be related to the change in the morpho-functional phenotype of C-28/I2 and T/C-28a4 cells incubated in DCC or -S medium. In fact, in both experimental conditions, induced marked

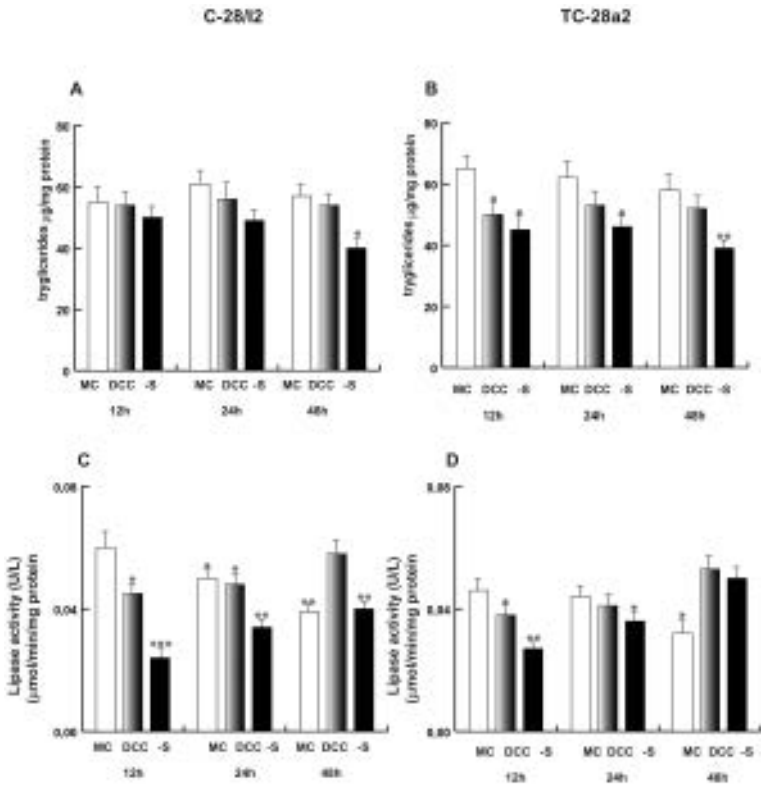


Fig. 5. *Starvation induced a reduction in lipids in C-28/I2 and T/C-28a4 cells.* ***A, B****): Triglyceride assay. The columns represent the mean ± SEM;* ***C, D****): Lipase activity assay. The columns represent the mean ± SEM. *P<0.05, **P=0.02 and ***P<0.02 versus MC at 12 h.*

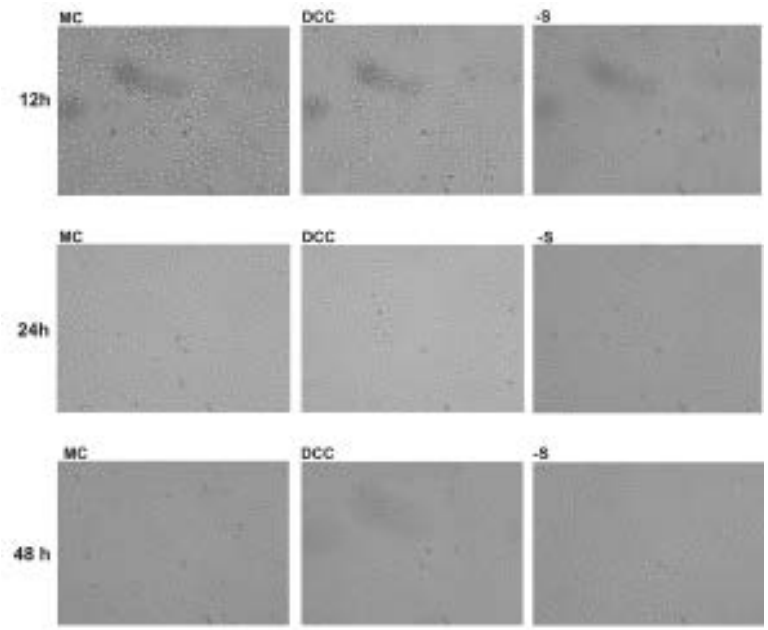


Fig. 6. *Starvation changes morphological features in the C-28/I2 cell line. Morphological features of C-28/I2 cells at 12, 24 and 48 h under different experimental conditions (original magnification: x 100). Each panel shows the result of one representative experiment repeated at least three times.*

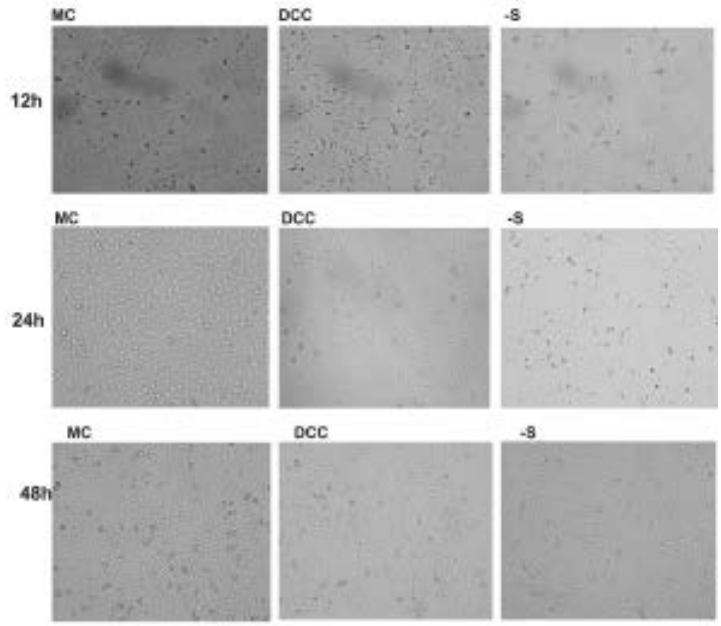


Fig. 7. *Starvation changes morphological features in the T/C-28a4 cell line. Morphological features of T/C-28a4 cells at 12, 24 and 48 h under different experimental conditions (original magnification: x 100). Each panel shows the results of one representative experiment repeated at least three times.*

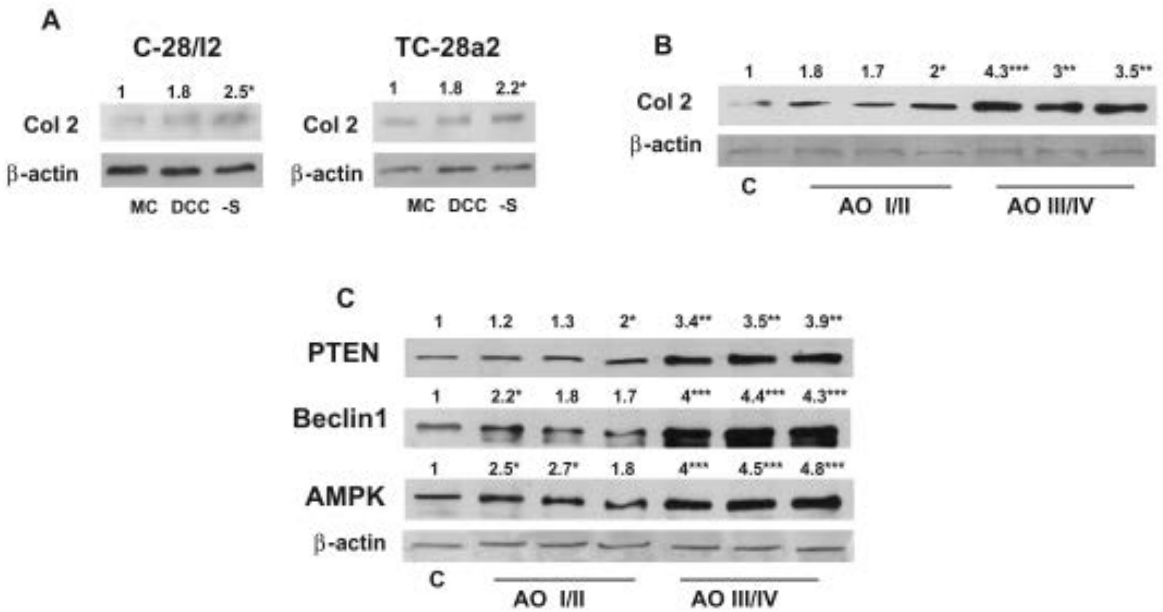


Fig. 8*. PTEN, Beclin1, AMPK and type II collagen profiles in cartilage samples from healthy and osteoarthritic patients.* ***A****): Immunoblot analysis of collagen II (Coll 2) in C-28/I2 and T/C-28a4 cells in MC, DCC, or -S medium at 24 h.* β-actin was used as a loading control*;* ***B****): Immunoblot analysis of collagen II in cartilage samples from healthy (****C****) and OA patients;* ***C****): Immunoblot analysis of PTEN, Beclin1 and AMPK in cartilage tissues from healthy (****C****) and OA patients.* β-actin was used as a loading control*. The autoradiographs show the results of one representative experiment and the numbers on the top of the blot are means of three independent experiments in which band intensities were evaluated in terms of arbitrary units of optical density and expressed as fold over control (****C****), which was assumed to be 1; *P<0.05, **P<0.02 and ***P<0.01 versus C.*

changes in cell morphology, with the appearance of elongation or stretching, whereas the cells cultured in MC appeared polygonal. The effects were more evident in the C-28/I2 cells (Fig. 6) compared to the T/C-28a4 cells (Fig. 7).

PTEN, Beclin1, AMPK and collagen II expression profiles in cartilage samples from patients with osteoarthritis (OA)

In OA, the stability of collagen fibrils is compromised, with extensive proteolytic breakdown of type II collagen, one of the major components of the extracellular matrix of articular cartilage, leading to cartilage erosion and joint deterioration.

Therefore, we analyzed collagen II expression by immunoblotting in chondrocytes cultured in the different media and we found that it was increased by starvation in both chondrocyte cell lines (Fig. 8A). Consistently, an increase in the expression of this cartilage degradation biomarker (30) was observed in patients with III/IV and I/II grade OA (Fig. 8B).

Based on the consistent evidence regarding PTEN, AMPK and autophagy and on the fact that there is metabolic impairment during nutrient depletion in the human chondrocyte cell lines, healthy and OA human cartilage samples were examined. As shown in Fig. 8C, PTEN, Beclin1 and AMPK expression was increased in the cartilage from OA patients compared with healthy donors. Specifically, these factors were more highly expressed in the III/IV grade OA samples than in the I/II grade OA samples.

DISCUSSION

The aim of our study was to underline novel molecular targets in human chondrocytes that could regulate their survival and metabolic phenotype. Previous studies investigated hormonal influences on human articular cartilage metabolism providing variable results due to the uncontrolled sources of cartilage and insufficient numbers of cells obtained from random procedures. To gain further insights into a possible involvement of steroids in cartilage metabolism as well as to evidence their metabolic phenotype, we evaluated glucose and lipid metabolism, by using the immortalized chondrocyte cell lines, C-28/I2 and T/C-28a4 (16), cultured in MC, DCC and -S.

PTEN is the primary negative regulator of the PI3K/AKT pathway, a classical survival signaling pathway in cells. A role for PTEN in influencing osteoclast survival and differentiation has been reported (8). The results of our analysis of PTEN and AKT expression in two human chondrocyte cell lines cultured under starvation conditions were similar to those reported in the literature for other differentiated cells: the increase in PTEN was concomitant with a decrease in AKT expression. PTEN/PI3K/AKT signaling is also related to autophagy and it has been suggested that autophagy is an intermediate step in the chondrocyte life cycle (31).

Furthermore, it has been reported that chondrocyte starvation does not increase the number of autophagosomes as is commonly observed in other cell lines and the authors suggested that in this cell type, autophagy is a constitutive rather than an adaptive pathway (31). Autophagy in chondrocyte cells is a new topic, for which knowledge is only beginning to be accumulated. The detection of LC3 and Beclin1 has been the mainstay for autophagy detection. However, LC3 expression levels can vary markedly between different cell types and in response to different stresses and there is also concern that over-expression of tagged versions of LC3 to facilitate the detection of autophagy interferes with the process itself. Technically, western blotting for LC3-I and LC3-II is not easy to obtain because successful blotting for LC3-I and –II requires particular skill (32). Beclin1 may be a more generally robust approach to determining the importance of autophagy in an experimental system because it has a central role in the regulation of the autophagy process. It forms a complex with other autophagins such as Ambra1, which binds to Beclin1, enhancing autophagosome formation (33).

Our data showed that Beclin1 and AMBRA levels increased during both types of starvation. It appears that the autophagic response serves to maintain the viability of cells because even if their number diminished with respect to those treated with MC, they continued to survive. The changes in the markers of survival and autophagy were not time dependent, however, the starved cells continued to survive and proliferate, most likely due to an adaptation to the environmental conditions.

It has been reported that chondrocyte activation

of autophagy causes increased catabolic signals and decreased anabolic signals (12). OA is also characterized by the progressive degradation and loss of articular cartilage due to unbalanced metabolic activities in chondrocytes (14). A difference in chondrocyte metabolism might be presumed when the cells have to adapt to the absence of growth factors. Typically, in cellular metabolism, glucose is metabolized via glycolysis into pyruvate, which then enters the tricarboxylic acid (TCA) cycle and is oxidized to produce ATP. In the absence of serum, metabolic deregulation may switch from glycolysis to mitochondrial respiration or *vv* and the conditions that trigger this switch need to be defined. Our results showed that C-28/I2 and T/C-28a4 cells have adapted to possess lower oxidative phosphorylation activity and consume less oxygen.

In other words, they switch to glycolytic metabolism, acquiring some features of the Warburg effect (WE). It has been reported that mouse chondrocytes are highly glycolytic cells and require a regular supply of glucose for optimal ATP production (34, 35). Even modest changes in the concentrations of glucose in the extracellular microenvironment of chondrocytes could impair IGF-I-mediated anabolic activity and thus promote a variety of joint pathologies (36). Therefore, steady physiological levels of glucose are critical for chondrocyte viability. We observed that the cellular concentrations of glucose increased upon starvation in both cell lines indicating that glucose utilization was reduced. This concept is supported by our observation that the activity of G6PDH, a key enzyme of the PPP that together with glycolysis represents the main pathway of glucose utilization, significantly declined in the starved cells. To our knowledge no notable observations about this enzyme have been reported in chondrocytes.

The metabolic results are consistent with PTEN elevation, accompanied by the repression of PI3K/AKT signaling and agree with a recent study reporting that cells that overexpress PTEN show reduced PI3K activity and impaired glucose metabolism (37).

Nutritional supplements, vitamins and nutraceuticals may be of benefit in the treatment of OA (38) and it is feasible that improved nutritional support of chondrocytes will prevent and alleviate OA. Therefore any data identifying the effects of nutrition on chondrocyte physiology will have an important impact on the prevention and treatment of a range of pathological conditions of articular cartilage.

In addition, a decline in G6PDH activity indicates a decline in ATP production. Because chondrocyte matrix synthesis is modulated by the balance between ATP generation and consumption, the mechanisms by which chondrocytes generate energy is a topic of interest. In this regard, articular chondrocytes are specialized for survival in an avascular environment and rely on long diffusion pathways to obtain O2 for oxidative phosphorylation.

Consistent with this specialization, cultured articular chondrocytes have a particularly active rate of anaerobic glycolysis (14). In fact, it was reported that OXPHOS may account for up to one-fourth of total steady-state ATP production within articular cartilage (39). In OXPHOS, electron transport is coupled with four enzyme complexes (CI–CIV) that are located in the mitochondrial inner membrane, with the exception of CII and ATP is synthesized at complex V (ATP synthase). Our data showed that C-28/I2 and T/C-28a4 cells weakly express CIV, indicating that OXPHOS works less efficiently in these cells than in cells that possess all five complexes, which supports the glycolytic phenotype reported for chondrocytes.

It is known that AMPK stimulates energy production from glucose and fatty acids during stress (40). There are emerging studies indicating that the function of AMPK is not restricted to the maintenance of energy metabolism during increased energy consumption but can also coordinate several housekeeping mechanisms, e.g., the autophagocytosis of damaged structures (41). In this manner, AMPK allows cells to adjust to changes in cellular energy demand and it was reported that AMPK is related to chondrocyte autophagy in mice (42). It has been shown that functional AMPK is required for starvation-mediated autophagy in growth plate chondrocytes (43).

From our study, AMPK content increased in both C-28/I2 and T/C-28a4 cells, particularly in the S-cultured cells, in which the expression of autophagic markers increased. The bioenergetic and biosynthetic requirements of cells are balanced by regulation of the flux of pathways that metabolize lipids other than glucose. Interestingly, in our study,

starvation induced a reduction in lipid content that was consistent with the observed increases in lipase activity and autophagy.

It is known that culturing chondrocytic cells in a monolayer leads to a process of dedifferentiation, whereby the cells acquire fibroblastic morphology, loosing their chondrocytic properties. From our observations, C-28/I2 and T/C-28a4 cells change their morphological phenotype, both in DCC and -S medium at 48 hours and continue to survive, further suggesting that they undergo metabolic reprogramming. Accordingly, glucose deprivation sensed by AMPK initiates different responses in oncogene-transformed fibroblasts, which die of apoptosis, whereas normal fibroblasts cease growth and division but do not die (44).

The effects of PTEN loss have been measured primarily in cancer cell lines that harbor numerous mutations and have lost their epithelial phenotype (45). Recent studies have reported that PTEN ablation in the cartilage of developing mice produces defects in growth plate organization and increased chondrocyte differentiation (46). Other investigators have shown that deleting PTEN in mature osteoblasts results in increased bone mass that accumulates throughout the animal's life span (47). Recently, it has been shown that PTEN elevation triggers systemic metabolic reprogramming (37). Taken together, the data from our experimental conditions of starvation showed that AMPK and PTEN elevation might favor the switch to a different metabolic and morphological phenotype. Parallel analyses of starved cells and OA cartilage tissues showed enhanced type II collagen degradation, as reported in the literature (30). Recently, elevated expression of PTEN (48) and Beclin1 (33) has been reported in OA chondrocytes. Our data from OA cartilage tissue samples revealed that increased PTEN, AMPK and autophagy, accompanied by metabolic dysfunction, are associated with augmentation of type II collagen degradation, supporting the hypothesis that the absence of certain nutrient(s) in cultured cells may resemble OA conditions.

Since diet or hormonal milieu are possible contributing factors to the pathogenesis of OA, future progress in treating degenerative joint disorders will be highly dependent on a better understanding of the unique nutritional requirements of chondrocytes. In this regard, clearer understanding of chondrocyte metabolic pathways may reveal the underlying metabolic disturbances that are directly responsible for cartilage degradation in OA and other arthropathies.

In this paper we report a novel aspect of chondrocyte biology through which PTEN and AMPK elevation induces the early reduction of chondrocyte survival through autophagy and induces metabolic reprogramming that in turn may influence the morphological phenotype of chondrocytes.

This knowledge may lead to new approaches and novel therapies to prevent and treat degenerative joint disease, enhancing approaches for the discovery and design of drugs or nutrients capable of modifying the course of these diseases.

ACKNOWLEDGEMENTS

This work was supported by MIUR Ex 60 % - 2014.

REFERENCES

1. Martel-Pelletier J, Wildi LM, Pelletier JP. Future therapeutics for osteoarthritis. Bone 2012; 51:297-311.
2. Hatori M, Klatte KJ, Teixeira CC, et al. End labeling studies of fragmented DNA in the avian growth plate: evidence of apoptosis in terminally differentiated chondrocytes. J Bone Miner Res 1995; 10:1960-8.
3. Adams CS, Horton WE. Chondrocyte apoptosis increases with age in the articular cartilage of adult animals. Anat Rec 1998; 250:418-25.
4. Kim HA, Song YW. Apoptotic chondrocyte death in rheumatoid arthritis. Arthritis Rheum 1999; 42:1528-37.
5. Goggs R, Carter SD, Schulze-Tanzil G, et al. Apoptosis and the loss of chondrocyte survival signals contribute to articular cartilage degradation in osteoarthritis. Vet J 2003; 166:140-58.
6. Hashimoto S, Ochs RL, Komiya S, et al. Linkage of chondrocyte apoptosis and cartilage degradation in human osteoarthritis. Arthritis Rheum 1998; 41:1632-38.
7. Zermeño C, Guzmán-Morales J, Macotela Y, et al.

Prolactin inhibits the apoptosis of chondrocytes induced by serum starvation J Endocrinol 2006; 189:R1-R8.
8. Guntur AR, Reinhold MI, Cuellar J Jr, et al. Conditional ablation of Pten in osteoprogenitors stimulates FGF signaling. Development 2011; 138:1433-44.
9. Van den Berg WB. Osteoarthritis year 2010: pathomechanisms. Osteoarthritis Cartilage 2011; 19:338-41.
10. Stambolic V, Suzuki A, De la Pompa JL, et al. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. Cell 1998; 95:29-39.
11. Rosenbloom AL, Silverstein JH. Connective tissue and joint disease in diabetes mellitus. Endocrinol Metab Clin North Am 1996; 25:473-83.
12. Denko CW, Malemud CJ. Metabolic disturbances and synovial joint responses in osteoarthritis. Front Biosci 1999; 4:D686-93.
13. Tsezou A, Iliopoulos D, Malizos KN, et al. Impaired expression of genes regulating cholesterol efflux in human osteoarthritic chondrocytes. J Orthop Res 2010; 28:1033-9.
14. Mobasheri A, Vannucci SJ, Bondy CA, et al. Glucose transport and metabolism in chondrocytes: a key to understanding chondrogenesis, skeletal development and cartilage degradation in osteoarthritis. Histol Histopathol 2002; 17:1239-67.
15. Goldring MB. The role of the chondrocyte in osteoarthritis. Arthritis Rheum 2000; 43:1916-26.
16. Goldring MB. Immortalization of human articular chondrocytes for generation of stable, differentiated cell lines. In Sabatini M, Pastoureau P et al. editors. Cartilage and osteoarthritis vol.1: cellular and molecular tools. Totowa: Humana Press 2004; p 23-36.
17. Altman R, Asch E, Bloch D, et al. Development of criteria for the classification and reporting of osteoarthritis. Classification of osteoarthritis of the knee. Arthritis Rheum 1986; 29:1039-49.
18. Altman R, Alarcón G, Appelrouth D, et al. The American College of Rheumatology criteria for the classification and reporting of osteoarthritis of the hip. Arthritis Rheum 1991; 34:505-14.
19. Kellgren JH, Lawrence JS. Radiological assessment of osteo-arthrosis. Ann Rheum Dis 1957; 16:494-502.
20. Guido C, Panza S, Santoro M, et al. Estrogen receptor beta (ERβ) produces autophagy and necroptosis in human seminoma cell line through the binding of the Sp1 on the phosphatase and tensin homolog deleted from chromosome 10 (PTEN) promoter gene. Cell Cycle 2012; 11:2911-21.
21. De Amicis F, Guido C, Avena P, et al. The endocrinology of the Progesterone/Progesterone Receptor (PR) B and PRA may be involved in the pathophysiology of the varicocele condition: action of progesterone on capacitation and human sperm metabolism. Int J Androl 2011; 34:430-45.
22. Guido C, Perrotta I, Middea E, et al. Human sperm physiology: estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) influence sperm metabolism and may be involved in the pathophysiology of varicocele-associated male infertility. J Cell Physiol 2011; 226:3403-12.
23. Aquila S, Bonofiglio D, Gentile M, et al. Peroxisome proliferator-activated receptor (PPAR) gamma is expressed by human spermatozoa: its potential role on the sperm physiology. J Cell Physiol 2006; 209:977-86.
24. Aquila S, Guido C, Laezza C, et al. A new role of anandamide in human sperm: focus on metabolism. J Cell Physiol 2009; 221:147-53.
25. Panteghini M, Bonora R, Pagani F. Measurement of pancreatic lipase activity in serum by a kinetic colorimetric assay using a new chromogenic substrate. Ann Clin Biochem 2001; 38:365-70.
26. Sugatani T, Alvarez U, Hruska KA. PTEN regulates RANKL- and osteopontin-stimulated signal transduction during osteoclast differentiation and cell motility. J Biol Chem 2003; 278:5001-08.
27. De Amicis F, Guido C, Santoro M, et al. A novel functional interplay between Progesterone Receptor-B and PTEN, via AKT, modulates autophagy in breast cancer cells. J Cell Mol Med. 2014; 18:2252-65.
28. Luo Z, Zang M, Guo W. AMPK as a metabolic tumor suppressor: control of metabolism and cell growth. Future Oncol 2010; 6:457-70.
29. Bohensky J, Leshinsky S, Srinivas V, et al. Chondrocyte autophagy is stimulated by HIF-1 dependent AMPK activation and mTOR suppression. Pediatr Nephrol 2010; 25:633-42.
30. Elsaid KA, Chichester CO. Review: Collagen mark-

ers in early arthritic diseases. Clinica Chimica Acta 2006; 365 :8–77.
31. Sasaki H, Takayama K, Matsushita T, et al. Autophagy Modulates Osteoarthritis-Related Gene Expression in Human Chondrocytes. Arthritis Rheum 2012; 64:1920-28.
32. Sandra Barth, Danielle Glick and Kay F. Autophagy: assays and artifacts. J Pathol. 2010; 221: 117-24.
33. Eskelinen E-L, Saftig P. Autophagy: A lysosomal degradation pathway with a central role in health and disease. Biochimica et Biophysica Acta 2009; 179:3664-73.
34. Otte P. Basic cell metabolism of articular cartilage. Manometric studies. Z Rheumatol 1991; 50:304-12.
35. Lee RB, Urban JP. Evidence for a negative Pasteur effect in articular cartilage. Biochem J 1997; 321:95-102.
36. Lioté F, Orcel P. Osteoarticular disorders of endocrine origin. Baillieres Best Pract Res Clin Rheumatol 2000; 14:251-76.
37. Garcia-Cao I, Song MS, Hobbs RM, et al. Systemic elevation of PTEN induces a tumor-suppressive metabolic state. Cell 2012; 149:49-62.
38. Fillmore CM, Bartoli L, Bach R, et al. Nutrition and dietary supplements. Phys Med Rehabil Clin N Am 1999; 10:673-703.
39. Reinecke F, Smeitink JA, Van der Westhuizen FH. OXPHOS gene expression and control in mitochondrial disorders. Biochim Biophys Acta 2009; 1792:1113-21.
40. Steinberg GR, Kemp BE. AMPK in Health and Disease. Physiol Rev 2009; 89:1025-78.
41. Mihaylova MM, Shaw RJ. The AMPK signalling pathway coordinates cell growth, autophagy and metabolism. Nat Cell Biol 2011; 13:1016-23.
42. Bohensky J, Leshinsky S, Srinivas V, Shapiro IM. Chondrocyte autophagy is stimulated by HIF-1 dependent AMPK activation and mTOR suppression. Pediatr Nephrol 2010; 25:633-42.
43. Srinivas V, Bohensky J, Shapiro IM. Autophagy: a new phase in the maturation of growth plate chondrocytes is regulated by HIF, mTOR and AMP kinase. Cells Tissues Organs 2009; 189:88-92.
44. Zhaohui F, Hu W, De Stanchina E, et al. The Regulation of AMPK beta1, TSC2 and PTEN Expression by p53: stress, cell and tissue specificity and the role of these gene products in modulating the IGF-1-AKT-mTOR pathways. Cancer Res 2007; 67:304353.
45. Bowen KA, Doan HQ, Zhou BP, et al. PTEN loss induces epithelial-mesenchymal transition in human colon cancer cells. Anticancer Res 2009; 29:4439-49.
46. Hsieh SC, Chen NT, Lo SH. Conditional loss of PTEN leads to skeletal abnormalities and lipoma formation. Mol Carcinog 2009; 48:545-52.
47. Liu JL, Mao Z, LaFortune TA, et al. Cell cycle-dependent nuclear export of phosphatase and tensin homologue tumor suppressor is regulated by the phosphoinositide-3-kinase signaling cascade. Cancer Res 2007; 67:11054-63.
48. Iwasa K, Hayashi S, Fujishiro T, et al. PTEN regulates matrix synthesis in adult human chondrocytes under oxidative stress. J Orthop Res 2014; 32:231-37.

ENDOTHELIAL PROGENITOR CELL ADHESION, GROWTH AND CHARACTERIZATION ON TRABECULAR TITANIUM AND TRABECULAR TITANIUM COATED WITH COLLAGEN OR DECELLULARIZED ECM

G. GASTALDI[1,4], L. CALIOGNA[2], L. BOTTA[3], M. GHIARA[2] and F. BENAZZO[2,4]

[1]Department of Molecular Medicine, Human Physiology Unit, University of Pavia, Italy; [2]Department of Clinical-Surgical Sciences, Diagnostics and Pediatrics, University of Pavia, Operative Unit of Orthopaedics and Traumatology, Fondazione IRCCS Policlinico San Matteo, Italy; [3]Department of Biology and Biotechnology "Lazzaro Spallanzani," University of Pavia, Italy; [4]Centre for Health Technologies (CHT), University of Pavia, Italy

Adequate blood supply is essential for prosthesis osteointegration and bone healing as it supplies oxygen, nutrition and progenitor cells. The bone healing process and vascularization depend upon the endothelial cells, which speed up implant osteointegration. Endothelial Progenitor Cells (EPC) are a population of stem cells that can reproduce, migrate and acquire mature endothelial phenotype. Their recruitment occurs in the tissue lesion to enhance neovascularization. Trabecular Titanium™ (TT™) is a new biomaterial with very interesting biomechanical characteristics and fast osteointegration. This study has investigated adhesion, proliferation and characteristics of EPC on three types of biomaterial: unmodified trabecular titanium, trabecular titanium coated with the ECM deposited by human mesenchymal stem cells isolated from subcutaneous adipose tissue and decellularized and trabecular titanium coated with type I collagen (control scaffold). MTT assay showed similar percentages of EPCs seeded on the different kinds of scaffold: 67% on TT, 70% on decellularized scaffolds and 82% on collagen-coated scaffolds. There were no statistically significant differences between the three groups. We therefore conclude that TT™ allows EPC adhesion and proliferation and, consequently, by permitting vascularization, it favours prosthesis osteointegration.

The biocompatibility of a prosthesis is essential when replacing a joint, but this factor alone is not enough to ensure long-term follow-up success. The design of the implant must allow for immediate stable fixation and osteointegration of the implant for later stability. The surface of the material plays a central role in the osteointegration process, as the bone needs to grow into the implant and give durable fixation. The interface between the prosthesis and the bone influences implant survival. In fact, in the past, this was one of the main causes of failure. Previously, cementation was the most common type of fixation in knee and hip arthroplasty, but the risk of cement debris and consequent loosening of the implant led to the development of new uncemented designs. In the last two decades, the uncemented prosthesis has grown in popularity, especially for young and high demand patients, increasing interest in the bone-prosthesis interface in terms of biomolecular studies and material design development. In hip arthroplasty, many studies report the success of uncemented stem and good results, especially in young patients

Key words: trabecular titanium, EPC, decellularized ECM, vascularization in bone tissue engineering

Mailing address: Matteo Ghiara,
Operative Unit of Orthopaedics and Traumatology,
Fondazione IRCCS Policlinico San Matteo,
Via Golgi, 19,
27100 Pavia, Italy
Tel.: +390382502854
e-mail: ghiaram@libero.it

0393-974X (2015)

(1). No such success has been reported in literature concerning knee arthroplasty (2, 3) and cementation remains the most popular type of fixation. There are more fixation problems with revision surgery as there are often bone defects that, if not treated, can compromise the success of the surgery.

The surgeon, therefore, needs an implant that can ensure immediate stability and durable osteointegration. Titanium and titanium alloys are commonly used in orthopedics and dental surgery due to their mechanical strength, chemical stability and biocompatibility. Trabecular titanium™ is a porous material like spongy bone, with high interconnectivity between the pores: this characteristic can induce in vitro osteogenic differentiation of human adipose-derived stem cells without osteogenic factors (4).

Adequate blood supply is essential for osteointegration and the bone healing process as it supplies oxygen, nutrition and progenitor cells.

The bone healing process and vascularization depend upon the endothelial cells, which speed up implant osteointegration (5). The interaction between the titanium implant surface and tissues influences implant survival in dental surgery (6). Inadequate vascular supply is often associated with reduced bone formation (7).

The first host tissue response to the implant is an inflammatory reaction. This is activated by surgical trauma and influenced by the presence of the implant: clot formation at the bone-prosthesis interface supports the subsequent healing process. Inflammation induces platelet activation, the migration and activation of inflammatory cells, vascularization, mesenchymal cell and osteoblast adhesion and protein synthesis. Endothelial cells express adhesion molecules depending on the material used (8). PECAM-1 adhesion molecules establish homophilic binding between neighboring endothelial cells and between themselves and the cytoscheleton (9).

Endothelial progenitor cells in type I collagen injected in vivo in immune-deficient mice can induce blood vessel formation (10). Furthermore, the collagen/heparin coating on the titanium surface increases EPC adhesion and the cells are smaller than those that adhere to an uncoated titanium surface: cell size and shape is inversely correlated with cell proliferation and migration speed (11). The endothelial cells in co-cultures adhere to extracellular matrix proteins produced by osteoblasts (12), including type I collagen.

EPCs are a population of stem cells, which can reproduce, migrate and acquire mature endothelial phenotype; their recruitment occurs in the tissue lesion to enhance neovascularization (13). This type of cell can be isolated from peripheral blood: after 12-15 days in culture they grow to form a colony of cells with a typical cobblestone appearance, they have high proliferative ability and they develop capillary-like structures (14, 15). EPCs can favor neovascularization in the presence of hypossic stimuli, i.e. they facilitate the de novo formation of blood vessels (16, 17, 18), which is different to angiogenesis where new vessels are formed from previous vessels by migration and proliferation of endothelial cells (19).

The aim of this study is to evaluate the ability of EPCs to adhere to and proliferate on the trabecular titanium surface, acquiring mature endothelial cell characteristics and their ability to interact with neighboring cells, expressing typical contact proteins like VE-cadherin and PECAM. We have also modified the titanium surface by covering it with type 1 collagen and de-cellularized extracellular matrix produced by osteoblasts. We have evaluated adhesion, proliferation, characterization and the interaction between EPCs and neighboring scattered cells. We selected type 1 collagen as its clinical use is FDA approved and we chose osteoblast extracellular matrix as its composition is the most similar to the natural one even when obtained by in vitro synthesis. The de-cellularized matrix produced by cellular secretion is a laboratory biomimetic surface with the same complexity as natural extracellular matrix.

MATERIAL AND METHODS

Cells

Human adipose derived stem cells (hASCs) were isolated from subcutaneous adipose tissue obtained from healthy donors during hip replacement surgery after informed consent (4).

The Ethic Committee of the Fondazione IRCCS Policlinico San Matteo of Pavia approved the study and informed consent from all subjects was obtained. Blood samples were obtained from healthy human volunteers or patients with fracture admitted to the Emergency

Department of the hospital. EPCs were isolated according to Dragoni et al (20). The endothelial lineage of the ECFCs was confirmed by FACS (fluorescent activated cell sorting), via the assessment of capillary-like networks in an "in vitro" Matrigel™ assay and by immunostaining with anti-CD31, anti-VWF, anti-VEGFr.

In Vitro Tube Formation Assay

Early passage (P2-P3) ECFCs were detached by trypsinization and resuspended in endothelial medium. Cells were plated at $2x10^4$ per well in Matrigel™ (BD Biosciences) coated 96-well plates and incubated at 37°C with 5% CO_2. Capillary network formation was progressively assessed from 1 to 4 h after seeding. Each EPC population was assessed for tube formation before being used for experiments.

Scaffolds

TT™ collagen coated scaffolds were seeded with hASC, cultured and differentiated as previously reported (4). Briefly, the hASC were seeded onto the scaffold and allowed to penetrate the structure for 2 hours before the growth medium was added. After 7 days, the osteogenic medium was added to induce osteogenic differentiation. Fourteen days after differentiation, the constructs were washed with phosphate buffered saline (PBS). Freeze-thaw cycling plus NH_4OH aqueous solution and DNase treatment was used for the decellularization. The freeze-thaw cycles (21) were repeated 6 times. The constructs were then immersed in an aqueous solution of 25 mM ammonia for 30 min on a slow-moving shaker. After the ammonia treatment, the constructs were washed 6 times with Milli-Q water and then treated with 70U/ml DNase I solution (Applichem) at 37°C for 90 min on a slow-moving shaker. After decellularization, the construct was thoroughly washed and then left to dry for 24 h.

TT™ collagen coated scaffolds were immersed in a 50 microgram/ml solution of Type I Collagen (Sigma-Aldrich) for 2 hours at room temperature. The scaffolds were then washed with Milli-Q water and left to dry.

Characterization of the ECM-coated scaffolds was performed using scanning electron microscope (SEM) and immunofluorescence staining. The scaffolds were coated with a thin evaporated layer of gold and observed using a Zeiss scanning electron microscope (SEM), mod. EVO MA10 (Carl Zeiss, Oberkochen, Germany). To evaluate the removal of cellular components, the constructs were stained with Hoechst (Sigma-Aldrich) for cell nuclei. The presence of type I collagen of ECM after decellularization was evaluated by immunofluorescence staining.

Immunofluorescence

For the immunofluorescence study EPCs seeded and grown on coverslips and the constructs were fixed with 3% paraformaldehyde for 30 min. All the samples were washed twice in PBS, blocked for 30 min with 1% BSA/0.02% Tween 20 and rinsed with PBS. They were then incubated with anti-type I collagen primary antibodies (Prestige Antibodies, Sigma-Aldrich, St. Louis, MO), anti-CD31 and anti-VE-cadherin (Santa-Cruz), anti-VWF and anti-VEGFr (Abcam), with a dilution equal to 1:100 in PAT (PBS containing 1% (w/v) bovine serum albumin and 0.02% (v/v) Tween 20 overnight at 4°C. Negative controls were incubated overnight at 4°C with PAT instead of the primary antibody. After three washes with PBS, all the samples were incubated for 30 min with Alexa 488 goat anti-rabbit IgG or Rhodamine Red-X-AffiniPure anti-mouse IgG (Li Starfish S.r.L) with a dilution equal to 1:1000 in PAT. At the end of incubation, the constructs were washed with PBS and then stained with DAPI (Sigma-Aldrich) to target cellular nuclei. The constructs were examined with a Nikon Eclipse 80i microscope (Nikon, Japan) equipped with digital image capture.

MTT assay

To evaluate the mitochondrial activity of the seeded cells on TT™ scaffolds during the culture period, a 3-(4, 5-dimethylthiazole-2-yl)-2.5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich) was performed on days 0, 7, 14 and 21. The culture medium was replaced with a 0.5 mg/ml solution of MTT in phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.4 mM KH2PO4, pH7.4) and the cell/scaffold constructs were incubated for 4 h in a humidified atmosphere of 95% air with 5% CO_2 at 37°C. Viable cells are able to reduce MTT into formazan crystals. After removing the MTT solution, 1 mL of isopropanol-HCl 0.1% was added to solubilize the formazan and the well plate containing the cultured 3D scaffold was agitated for 20 min on a shaker. Aliquots of 500 ml were sampled and the related absorbance values were measured at 570 nm. An appropriate standard curve was made for each experiment.

Molecular biology tests

RNA extraction and retrotranscription. RNA was extracted from cells with TRI reagent® RNA isolation Reagent (Sigma-Aldrich, Italy) to evaluate gene expression. After 21 days of culture, cells/scaffold constructs were washed with PBS and then put in a ß-mercaptoethanol and lysis buffer, frozen in liquid nitrogen and stored at -80°C until extraction. RNA extraction included the addition of DNase to increase the extraction purity. The total RNA extracted was then reverse transcribed into cDNA, with the M-MLV Transcriptase (Promega). Reverse transcription was performed according to Laforenza et al (22). RNA

of control cells was extracted from EPCs before they were seeded on TT scaffolds (control). The expression of von Willebrand factor (VWF) and endothelial NOS (e-NOS), which are indicators of EPC maturation (23), was evaluated by quantitative real-time (qPCR).

Quantitative real-time RT-PCR was performed in triplicate using 1 ml cDNA (obtained as indicated above) and specific primers for VWF (Hs_VWF_1_SG QuantiTect Primer Assay QT00051975, Qiagen) and e-NOS (Hs_NOS1_1_SG Quantitect Primer Assay QT 00043372, Qiagen). The QuantiFast-SYBR Green PCR Kit (Qiagen SpA, Milan, Italy) was used according to the manufacturer's instructions and qPCR was performed using Rotor Gene 6000 (Corbett). The conditions were as follows: initial denaturation at 95°C for 5 min; 40 cycles of denaturation at 95°C for 30 s; annealing at 58°C for 30 s and elongation at 72°C for 40 s. Melting curves were generated to detect the melting temperatures of specific products immediately after the PCR run. The qPCR reactions were normalized against the expression of the housekeeping gene beta-2-microglobulin (B2M) (Hs_B2M_1_SG, QuantiTect Primer Assay QT00088935, Qiagen). Relative mRNA levels were referred to the EPC (control) before seeding on scaffold (ΔΔCt). Fold change values were expressed as $2^{-\Delta\Delta Ct}$.

RESULTS

Characterization of EPC isolated from peripheral blood

Cells isolated from peripheral blood mononucleated by density gradient centrifugation over Histopaque formed primary colonies after 10-15 days in complete endothelial growth medium (EGM-2MV, Lonza) with the typical cobblestone morphology (Fig. 1B). These cells were trypsinized at third passage and flow cytometry analysis was performed to evaluate the expression of surface markers. They expressed the typical endothelial progenitor markers and were negative for mesenchymal stem cell markers (Fig. 1A). EPCs cultured for 4 h in multiwells coated with Matrigel demonstrated a change in morphology, connecting to each other to form tube-like structures (Fig. 1C). Immunofluorescence experiments demonstrated that the cells trypsinized at the third passage were positively stained by antibody anti-VWF and anti-VEGFr (Fig. 1D, E).

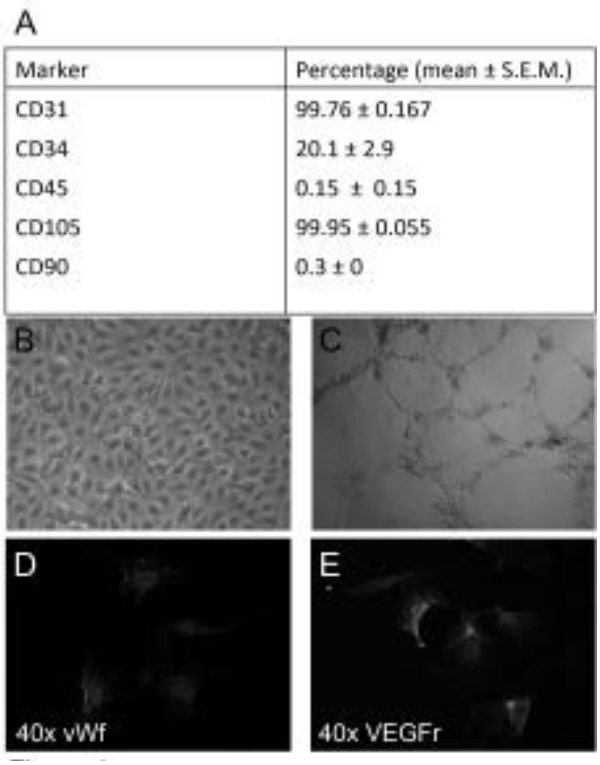


Fig. 1. *Characterization of EPC isolated from peripheral blood.* ***A****): Percentage of markers in EPC at passage 3 stage, media±S.E.M. of three different preparations;* ***B****): cobblestone-like morphology of EPC cultured in monolayer in EGM-2MV medium; magnification, 10x;* ***C****): tube formation on Matrigel™ after 4 h from seeding, magnification: 4x;* ***D, E****): immunofluorescence staining (green) of VWF(D) and VEGFr (E) of EPC in monolayer culture in EGM-2MV. Cell nuclei are stained with DAPI (blue). Images are representative pictures of wells seeded in triplicate using cells from 3 different donors. Magnification 20x.*

Scaffolds

hASCs seeded on TT™ scaffold secreted an abundant matrix containing type I collagen that surrounded many cells proliferated on the scaffolds (Fig. 2A and B, arrowheads). The decellularization method used was able to remove the cells without damaging the matrix: the immunofluorescence image shows the presence of type I collagen (Fig. 2C); the presence of the matrix on the scaffold after decellularization was shown by SEM analysis. (Fig. 2D, arrows).

Proliferation and characteristics of EPC on

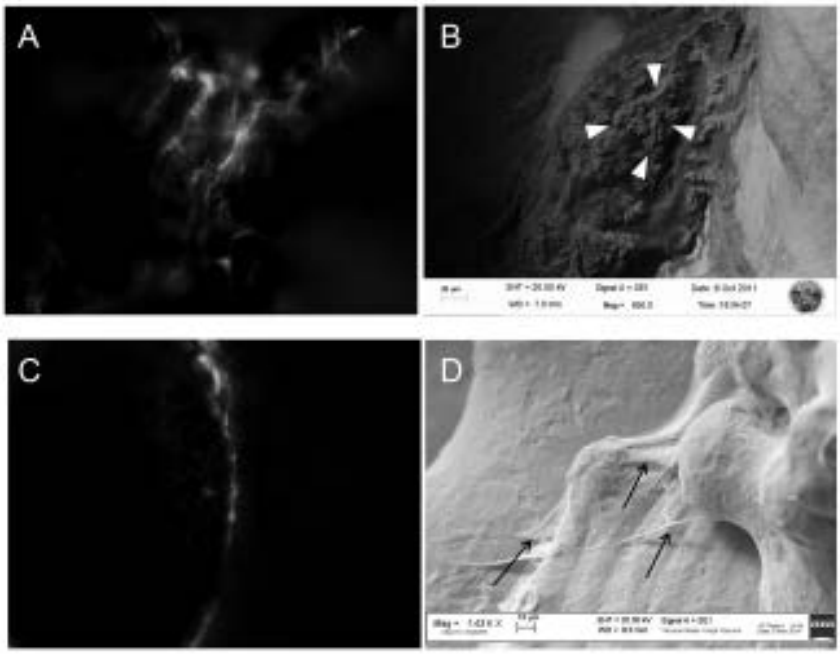


Fig. 2. *Characterization of decellularized ECM-coated scaffold. **A**, **B**): Images of Trabecular Titanium scaffold seeded with hASC and coated with the original ECM; (**C, D**): Images of Trabecular Titanium scaffold seeded with hASC and coated with the decellularized ECM; **A, C**): Immunostaining for type I collagen (**green**) shows that the decellularization procedure removes cells preserving the matrix. Nuclei are stained with Hoechst (**blue**), magnification 20x; **D**): Analysis with SEM confirms the absence of cells after decellularization with the presence of abundant ECM (arrows); **B**): Many cells are evident (arrowheads).*

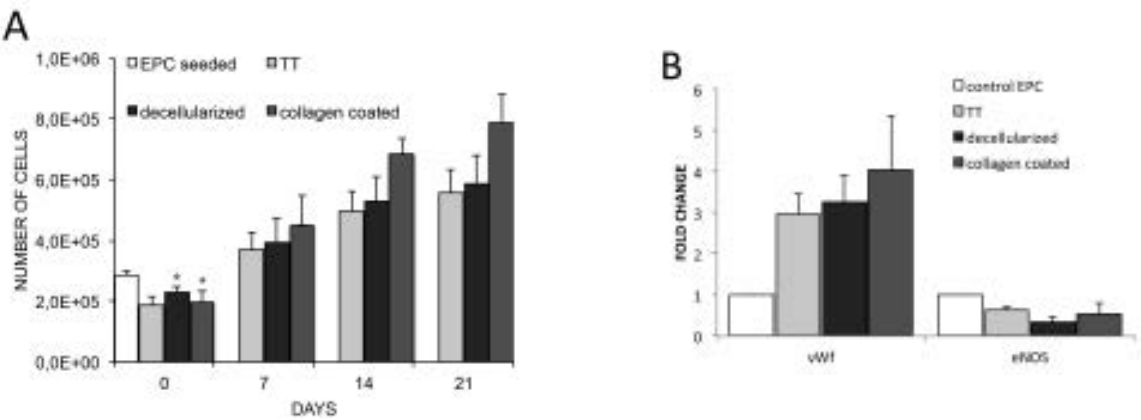


Fig. 3. *A): MTT assay on EPC seeded and grown on different scaffolds for 3 weeks. Each bar represents the mean ± SEM of four different preparations. *, p≤0.05 vs 7, 14 and 21 days (one-way ANOVA method followed by Bonferoni test; **B**): VWF and eNOS transcript expression in EPC grown on different scaffolds after 21 days from seeding. mRNA levels were measured by real-time RT-PCR relative to the beta-2-microglobulin internal standard (see Materials and Methods) and the values obtained were reported as DDCT (fold change) versus control EPC. Bars represent the mean ± S.E.M. of four different experiments, each from different RNA extracts.*

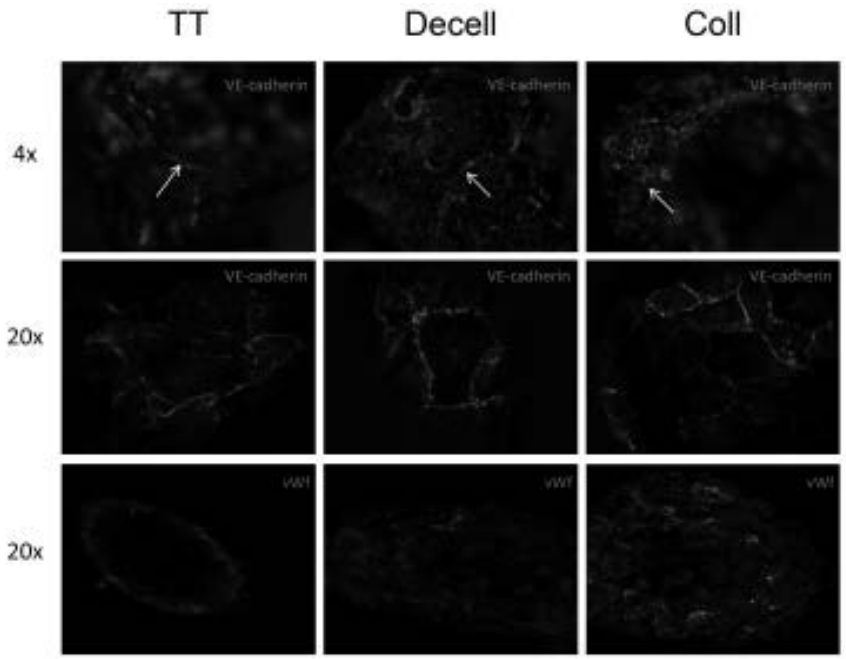


Fig. 4. *Immunostaining for VE-cadherin and VWF of EPC grown on trabecular titanium scaffold (**TT**), TT coated with decellularized extracellular matrix (**Decell**) and TT coated with type I collagen (**Coll**). Arrows show the presence of tubular structures. VE-cadherin is located at junctions between endothelial cells. VWF is localized in the cytoplasm of the cells. Magnifications: 4x and 20x.*

scaffolds – MTT test showed similar percentages of EPCs seeded on the different kinds of scaffold: 67% on TT, 70% on decellularized scaffolds and 82% on collagen-coated scaffolds. There were no statistically significant differences between the three groups. The time course of the EPC number on the scaffolds was similar: the cells proliferated throughout the experiment, being 2-3 times higher after 3 weeks of culture. For each kind of scaffold the number of cells at time 0 was statistically different to that of cells at 14 and 21 days of culture (Fig. 3A). The EPCs grown of the different scaffolds maintained the mRNA expression of VWF and eNOS, specific markers of endothelial cells expressed as fold change over EPC before seeding (Fig. 3B, control EPC).

Confocal microscope images confirm that the cells are positive for specific markers of endothelial cells VE cadherin and VWF. At low magnification (4X), it is possible to see tubular-like structures (Fig. 4, white arrows). At higher magnification (20X), the contacts between the cells are shown (high positivity for VE Cadherin) and appear as roundish structures.

Marking by anti-VWF shows that the cells are high positive for von-Willebrand factor, a specific protein secreted by mature endothelial cells: the marking is like lots of little spots in the cellular cytoplasm (Fig. 4).

SEM analysis

All the cells/scaffold constructs showed many cells positioned to form tubular-like structures (Fig. 5, arrows). Higher magnification showed (arrowheads) the cells connecting with one another (insert in Fig. 5, TT).

DISCUSSION

Orthopedics has seen an increased number of joint replacements in the last decades and their indications, especially for the hip and knee, have widened. Consequently, the number of revision cases has also increased and the new goal, in recent years, has been to develop new materials and designs to improve implant survival and patient outcome, both

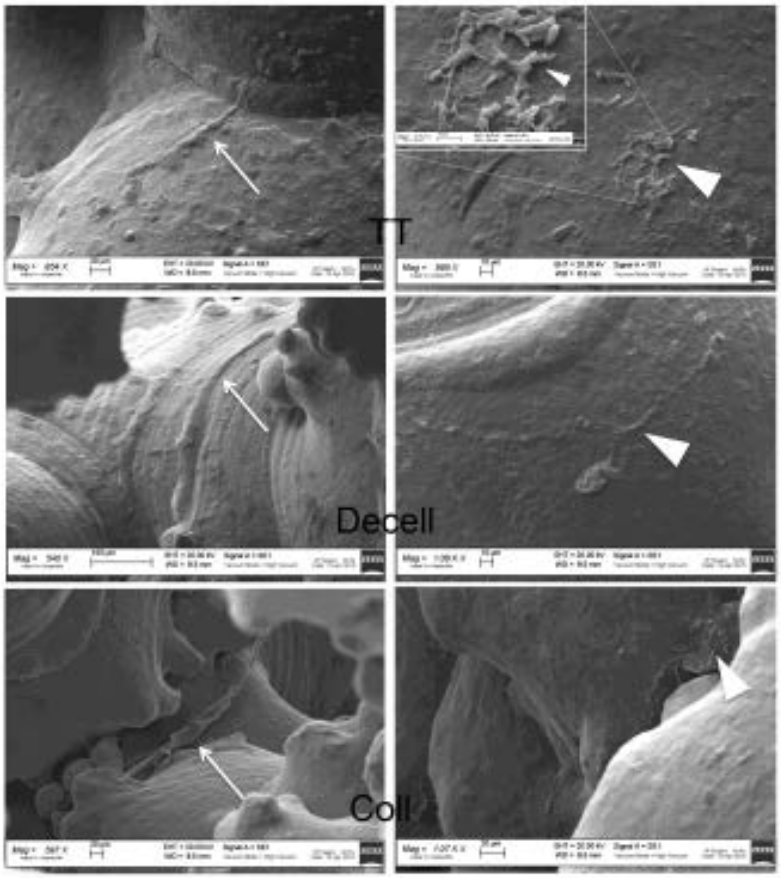


Fig. 5. *Scansion electron microscopy of trabecular titanium scaffold (TT), TT coated with decellularized extracellular matrix (Decell) and TT coated with type I collagen (Coll) seeded with EPC and cultured with EGM-2MV for 28 days. Capillary-like tubular structures are shown on each scaffold (arrows). Higher magnification shows cells connecting to each other.*

in old and young patients. The market has tried to develop new metal or metallic alloy or prosthesis surfaces permitting excellent fixation to bone without needing cementation to reduce the risk of wear and particle debris.

Cemented acetabular revision, for example, has been associated with high risk of loosening (24). In the last decade, the introduction of trabecular metal (TM™), (the benchmark of porous material based on tantalum deposition on a carbonate skeleton) in difficult primary and revision surgery has resulted in excellent osteointegration and biological fixation with increased depth of bone ingrowth and improved initial stability (24, 25). It also gave better results in terms of survivorship (26, 27) and subsequently initiated the development of further new materials. These results triggered further research into improved fixation methods and new metal structures, especially for use in cases with major bone loss (difficult primary revision surgery).

TT™ is a new biomaterial with a regular three-dimensional structure characterized by high open porosity that simulates trabecular bone (28). TT™ facilitates primary stability of the implant during surgery and secondary stability following bone ingrowth. Various studies have reported TT™ to have very good biomechanical characteristics (28) and fast osteointegration (4). The literature presents a study (29) involving 81 acetabular revisions performed using three types of acetabular TT™ cups and hemispherical module and augments. Steno et al. at a mean follow-up of 38 months, reported immediate

stability, bone healing, osteointegration of TT™ acetabular cup and absence of radiological signs of loosening in all but 4 cases: there was subsidence of the acetabular cup in 3 cases (that stabilized at six months) and the implant was unstable and required revision in 1 case. The Authors therefore concluded that TT™ cups facilitate reliable and reproducible biological fixation in acetabular revision surgery.

Another study (30) reports that another type of TT custom-made implant is useful in the case of acetabular revisions with a Paprosky type 3 defect; Baauw et al. compared planned and post-operative position and orientation of the custom-made implants in 16 patients by CT-scan. The results were encouraging.

Cementless implant survival depends on rapid vascularization, which allows engraftment and subsequently osteointegration. Many different cells are important for the bone healing process: endothelial progenitor cells, endothelial cells, osteoblasts and stem cells. Effective vascularization is essential to repair as it supplies oxygen, nutrients, cytochines and growth factors. Ideal biomaterial is cytocompatible, it interacts with surrounding tissues, gives chemical stability and mechanical strength and allows cellular adhesion and proliferation. In the field of cardiovascular tissue engineering, there has recently been increased interest in metal surfaces, which attract stem cells, particularly endothelial progenitor cells (31).

The aim of this study was to investigate adhesion, proliferation and characteristics of endothelial progenitor cells (EPC) on three kinds of biomaterials: unmodified trabecular titanium, trabecular titanium coated with the ECM deposited by human mesenchymal stem cells isolated from subcutaneous adipose tissue and decellularized and trabecular titanium coated with type I collagen (control scaffold). This latter scaffold was chosen as the characteristics of EPC growth on this type of matrix is well-known. The method used to isolate EPCs from peripheral blood involves the seeding of mononuclear cells obtained via gradient centrifuge on collagen-coated plates. The performance of trabecular titanium in clinical practice is well known. In vitro, it induces osteogenic mesenchymal stem cell differentiation even without osteogenic factors (4). The TT™ scaffold was coated with a natural matrix of mesenchymal stem cells to see if this could improve the material's performance in terms of vascularisation. The extracellular matrix was decellularized so it could be used at a later stage as well as in vivo. The decellularization method chosen meant the scaffold was still coated with a matrix rich in type I collagen, an essential component of the extracellular matrix (Fig. 2).

Scanning microscope confirmed the presence of the matrix and the absence of cells. No cells were seen even with high magnification, whilst numerous on the control scaffold coated with a non-decellularized matrix (Fig. 2B, D). After seeding on the collagenated plates, the EPCs displayed cobblestone morphology with cell-to-cell connections and expressed the two proteins characteristic of mature endothelial cells (von Willebrand factor and VEGFr). EPCs grown on the titanium structures maintained endothelial phenotype characteristics: they were still positive for von Willebrand factor after 3 weeks (Figure 4), which is in line with the results of Breithaupt-Faloppa and coworkers, had with umbilical cord endothelial cells which, however, were not positive for VE-caderina (32). As far as we are aware, this is the first time that cell interaction, shown by anti-VE-caderina antibody marking, has been demonstrated on a porous three-dimensional titanium structure (Fig. 4). The formation of a confluent layer of EPCs positive for CD31 was shown with in vivo experiments on pig following the seeding of cells expanded in vitro on a tubular titanium device (33).

The EPCs also grew and remained positive for CD31 on trabecular titanium scaffolds (data not shown). Scanning microscope images also appear to confirm that EPCs grown for 21 days on coated and uncoated trabecular titanium scaffolds connect to form three-dimensional tube-like structures within the matrix. In some areas, high magnification shows the sprouting of the cells delimiting a central cavity (Fig. 5, TT). This is similar to EPCs seeded on Matrigel, observed with contrast phase microscope (Fig. 1C). The propensity of EPCs to adhere to and proliferate on differently treated titanium surfaces (various chemicals to promote adhesion and improve performance) has been reported by other authors: EPCs grown on SLA titanium surfaces (sand-blasted, large-grit, acid-etched, i.e. more hydrophilic), express more VEGF than the other types of titanium

surfaces used (34). Real-time QT-PCR experiments confirmed that EPCs' expression of VWF mRNA and eNOS was similar to that of control cells before seeding on the scaffolds and there was no difference between the surfaces (Fig. 3B).

We can conclude that the immediate primary stability of the TT™ implants is a result of the metal's mechanical properties and the secondary stability is a consequence of osteointegration. The formation of new vessels is essential for bone healing and remodeling, so our study in vitro shows that TT™ allows EPC adhesion and proliferation. We can therefore state that vessel formation and bone integration are linked and that TT™ is a suitable surface for this interaction and consequently favors the osteointegration in cases of difficult surgery with severe bone loss.

ACKNOWLEDGEMENTS

The authors would like to thank Lima Corporate for the Trabecular Titanium scaffolds.

REFERENCES

1. Wechter J, Comfort TK, Tatman P, Mehle S, Gioe TJ. Improved survival of uncemented versus cemented femoral stems in patients aged <70 years in a community total joint registry. Clin Orthop Relat Res 2013 471: 3588-95.
2. Wang H, Lou H, Zhang H, Jiang J, Liu K. Similar survival between uncemented and cemented fixation prostheses in total knee arthroplasty: a meta-analysis and systematic comparative analysis using registers. Knee Surg Sports Traumatol Arthrosc 2014 22: 3191-7.
3. Gandhi R, Tsvetkov D, Davey JR, Moahomed NN. Survival and clinical function of cemented and uncemented prostheses in total knee replacement: A META-ANALYSIS. J Bone Joint Surg (Br) 2009; 91B:889-95.
4. Benazzo F, Botta L, Scaffino MF, Caliogna L, Marullo M, Fusi S, Gastaldi G. Trabecular titanium can induce in vitro osteogenic differentiation of human adipose derived stem cells without osteogenic factors. J Biomed Mater Res A 2014; 102(7):2061–71.
5. Schliephake H, Scharnweber D, Dard M, Sewing A, Aref A, Roessler S. Functionalization of dental implant surfaces using adhesion molecules. J Biomed Mater Res B Appl Biomater 2005; 73:88-96.
6. Kämmerer PW, Heller M, Brieger J, Klein MO, Al-Nawas B, Gabriel M. Immobilisation of linear and cyclic RGD peptides on titanium surfaces and their impact on endothelial cell adhesion and proliferation. Eur Cell Mater 2011; 21:364-72.
7. Glowacki J. Angiogenesis in fracture repair. Clin Orthop. Relat. Res 1998; 355S:82-9.
8. Pu FR, Williams RL, Markkula TK, Hunt JA. Effects of plasma treated PET and PTFE on expression of adhesion molecole by human endothelial cells in vitro. Biomaterials 2002; 23:2411-28.
9. Albelda SM, Buck CA: Integrins and other cell adhesion molecole. FASEB J. 1990; 4:2868-80.
10. Allen P, Melero-Martin J, Bischoff J. Type I collagen, fibrin and PuraMatrix matrices provide permissive environments for human endothelial and mesenchymal progenitor cells to form neovascular networks. J Tissue Eng Regen Med 2011; 5:e74-e86.
11. Chen J, Chen C, Chen Z, Chen J, Li Q, Hunag N. Collagen/heparin coating on titanium surface improbe the biocompatibility of titanium applied as a blood-contacting biomaterial. J Biomed Mater Res A 2010; 95A:341-9.
12. Duttenhoefer F, Lara de Freitas R, Meury T, et al. 3D scaffold co-seeded with human endothelial progenitor and mesenchymal cells: evidence of prevascularization within 7 days. Eur Cell Mater 2013; 26:49-65.
13. Pompilio G, Capogrossi MC, Pesce M, et al. Endothelial progenitor cells and cardiovascular homeostasis: Clinical implications. Int J Cardiol 2009; 131:156-67.
14. Mead LE, Prater D, Yoder MC and Ingram DA. Isolation and characterization of endothelial progenitor cells from human blood. Curr Protoc Stem Cell Biol 2008; Chapter 2:Unit 2C1.
15. Critser PJ, Yoder MC. Endothelial colony-forming cell role in neoangiogenesis and tissue repair. Curr Opin Organ Transplant 2010;15:68-72.
16. Krenning G, van Luyn MJ, Harmsen MC. Endothelial progenitor cell-based neovascularization: implications for therapy. Trends Mol Med 2009; 15:180-9.

17. Dome B, Dobos J, Tovari J, Paku S, Kovacs G, Ostoros G, Timar J. Circulating bone marrow-derived endothelial progenitor cells: characterization, mobilizationand therapeutic considerations in malignant disease. Cytometry Part A 2008; 73:186-93.
18. Yamahara K, Itoh H. Potential use of endothelial progenitor cells for regeneration of the vasculature. Ther Adv Cardiovasc Dis 2009; 3:17-27.
19. Risau W. Mechanisms of angiogenesis. Nature 1997; 386:671-4.
20. Dragoni S, Laforenza U, Bonetti E, et al. Vascular Endothelial Growth Factor Stimulates Endothelial Colony Forming Cells Proliferation and Tubulogenesis by Inducing Oscillations in Intracellular Ca^{2+} Concentration. Stem Cells 2011; 29:1898-907.
21. Lu H, Hoshiba T, Kawazoe N, Chen G. Autologous extracellular matrix scaffolds for tissue engineering. Biomaterials 2011; 32:2489-99.
22. Laforenza U, Miceli E, Gastaldi G, Scaffino MF, Ventura U, Fontana JM, Orsenigo MN, Corazza GR. Solute transporters and aquaporins are impaired in celiac disease. Biol Cell 2010; 102:457-67.
23. Cui X, Zhang X, Guan X, Li H, Lu H, Cheng M. Shear stress augments the endothelial cell differentiation marker expression in late EPCs by upregulating integrins. Biochem Biophys Res Commun 2012; 425(2):419-25.
24. Pulido L, Rachala SR, Cabanela ME. Cementless acetabular revision: past, present and future Revision total hip arthroplasty: the acetabular side using cementless implants. Int Orth 2011; 35:289-98.
25. Bobyn JD, Poggie RA, Krygier JJ, et al. Clinical validation of a structural porous tantalum biomaterial for adult reconstruction. J Bone Joint Surg Am 2004; 86-A(2):123-9.
26. Unger AS, Lewis RJ, Gruen T. Evaluation of porous tantalum uncemented acetabular cup in revision total hip arthroplasty: clinical and radiological results of 60 hips. J Arthroplasty 20:1002-9.
27. Abolghasemian M, Tangsataporn S, Sternheim A, Backstein D, Safir O, Gross AE. Combined trabecular metal acetabular shell and augment for acetabular revision with substantial bone loss: a mid-term review. Bone Joint 2013; 95-B(2):166-72.
28. Marin E, Fusi S, Pressaco M, Paussa L, Fedrizzi L. Characterization of cellular solids in TiAl4V for orthopaedic implant applications: trabecular titanium. J Mech Behav Biomed Mater 2010; 3:373-81.
29. Boris Steno, Milan Kokavec, Libor Necas. Acetabular revision arthroplasty using trabecular titanium implants, International Orthopaedics 2015; 39:389-95.
30. Baauw M, Van Hellemondt GG, Van Hooff M, Spruit M. The accurracy of positioning of a custom-made implant within a large acetabular defect ar revision arthroplasty of the hip. Bon Joint J 2015; 97-B(6):780-5.
31. Moradi I, Behjati M, Kazemi M. Application of anodized titanium for enhanced recruitment of endothelial cells. Nanoscale Res Lett 2012; 7(1):298.
32. Breithaupt-Faloppa AC, Tavares de Lima W, Oliveira-Filho RM, Kleinheinz J. In vitro behaviour of endothelial cells on a titanium surface. Head Face Med 2008; 4:14.
33. Jantzen AE, Lane WO, Gage SM, et al. Autologous endothelial progenitor cell-seeding technology and biocompatibilità testing for cardiovascular devices in large animal model. J Vis Exp 2011; 55:e3197.
34. Ziebart T, Schnell A, Walter et al. Interactions between endothelial progenitor cells (EPC) and titanium implant surfaces. Clin Oral Invest 2013; 17:301-9.

BIOPHYSICAL STIMULATION FOR NONUNIONS

E. DELLA BELLA[1,2], M. TSCHON[1], C. STAGNI[3], D. DALLARI[3] and M. FINI[1,4]

[1]Laboratory of Preclinical and Surgical Studies, Rizzoli Orthopedic Institute, Bologna, Italy; [2]Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Bologna, Italy; [3]Conservative Orthopedic Surgery and Innovative Techniques Ward, Rizzoli Orthopedic Institute, Bologna, Italy; [4]Laboratory of Biocompatibility, Innovative Technologies and Advanced Therapies, Department Rizzoli RIT, Rizzoli Orthopedic Institute, Bologna, Italy

Nonunions account for 5-10% on the total number of fractures. Biophysical stimulation is a non-surgical, conservative, frequently used therapy in nonunions and a greater efficacy has been demonstrated for pulsed electromagnetic fields (PEMF). The mechanisms of action of PEMF at cellular and molecular levels are still under debate and no dose-response study is available. Moreover, the vast majority of *in vitro* studies were conducted on healthy cells. The primary aim of the research was to investigate the capacity of PEMF with different exposure times to stimulate the osteogenic process in cells from the callus of a nonunion patient. Another important objective was the characterization of nonunion cells in terms of clonogenicity, cluster of differentiation expression and the tri-lineage differentiation capacity. Overall, the results indicated the presence of osteochondroprogenitor cells in the callus of a nonunion, with an impairment in the osteogenic differentiation process. PEMF may enhance cell viability, the formation of osteoid matrix and accelerate the process of osteogenic differentiation. BMP-4 production, *TIMP1* and *TIMP2* expression were influenced, as well as *VEGFA*, whose early upregulation may account for a possible improvement in both the osteogenic and vasculogenic processes. In conclusion, even with some discussed limitations, these preliminary data showed the presence of a multipotent progenitor population and suggested some hints of the effect of PEMF on nonunion cells.

The consolidation of a fracture occurs through two general mechanisms: primary bone healing that derives from mechanical stabilization of fractures and secondary bone healing that is prevalently biological through the formation of the callus. Secondary healing begins with an inflammatory phase in which mediators are released by the injured bone during bleeding and haematoma formation. They include Platelet-derived growth factor (PDGF), interleukin-6 (IL-6), IL-1 and prostaglandin E_2 (PGE_2), which recruit macrophages and mesenchymal stem cells and promote vascular proliferation.

After the formation of a soft callus, vascular ingrowth together with both endochondral and intramembranous ossification leads to the formation of a hard callus. The last phase of fracture healing consists of remodeling, when the initial woven bone is reabsorbed and lamellar bone is deposited (1-3). The process of fracture healing depends on a wide range of surgery-, fracture- and patient-related factors. The percentage of delayed unions, i.e. fractures whose recovery is slower than it should

Key words: nonunion tissue, pulsed electromagnetic fields (PEMF), multipotent progenitor cells; biophysical stimulation

Mailing address: Dr. Matilde Tschon,
Laboratory of Preclinical and Surgical Studies,
Rizzoli Orthopedic Institute,
Via di Barbiano 1/10,
40136 Bologna, Italy
Tel.: +390516366558 Fax: +390516366580
e-mail: matilde.tschon@ior.it

0393-974X (2015)

be, is comprised between 3% and 12% (4-6), while nonunions, i.e. fractures whose healing is absent and union cannot occur without further intervention, account for 5-10% on the total number of fractures (7,8). FDA has recently suggested that any fracture, failing to heal at more than 6 months after trauma, should be considered nonunions.

The microenvironment plays a major role in the biology of the fracture nonunion, as indicated by Frost in 1989 (9). The unbalance of several growth factors could play a major contribution in the biological development of nonunions, especially those related to angiogenesis and bone anabolic processes. Zimmermann et al. (10) reported a decrease in the systemic levels of Transforming growth factor beta 1 (TGF-β1) in delayed union patients and the lack of expression of both Bone morphogenetic protein 2 (BMP-2) and BMP-4. The same author in 2012 (11) performed a deep analysis of gene expression in fracture callus samples and found the upregulation of 8 out 226 genes involved in osteo- and chondrogenesis, including *TSC22*, which is a component of the TGF-β signaling pathway. In the nonunion tissue Fajardo and coworkers found higher levels of BMP-4, Drm/Gremlin, follistatin and Noggin and a reduction of BMP-7 in comparison to the healing bone (12).

Other systemic alterations of growth factors include a decrease in serum levels of basic Fibroblast Growth Factor (bFGF) and PDGF-AB (13) and lower serum concentrations of components of the Growth Hormone (GH)/Insulin-like Growth Factor 1 (IGF-1) axis (14) in atrophic nonunions. Surprisingly, no significant differences were detected in Vascular Endothelial Growth Factor (VEGF) serum levels of impaired and normal fracture healing patients (15). Mathieu et al, instead, found a decrease of Stem Cell Factor (SCF), PDGF-BB, Matrix Metalloproteinase 2 (MMP-2) and IGF-1 in the serum of nonunion patients, while leptin and IL-6 were increased (16).

Also MMPs and their inhibitors (Tissue Inhibitors of Metalloproteinases, TIMP) play an important role in bone repair and remodeling. MMP-7 and MMP-12 were significantly upregulated in hypertrophic nonunion tissue (17). Both MMPs were also responsible for BMP-2 degradation. The levels of circulating MMPs were measured in union and atrophic nonunion patients, observing an increase in proMMP-1 and MMP-8 and a decrease in TIMP-1 concentration (18). However, from these literature data, any biological marker or panel of markers that could be related to the onset of nonunions, could not be identified.

The costs associated with nonunions are extremely high (accounting in the USA for $23.000-58.000) because they comprise initial surgery with grafting, frequent clinical and radiographic assessments, affecting patients' quality of life and indirect costs related to missed working days (2, 19). The treatment of nonunions include non-surgical approaches, surgical interventions or a combinations of both and treatment strategies are highly personalized (20). Primarily, the strategy should restore alignment, insure appropriate stability, enhance fracture biology and eradicate infections, if any. Moreover, autologous iliac crest transplants, demineralized allogeneic bone matrix, bone morphogenetic proteins, such as BMP-7 (approved from the Food and Drug Administration in 2001), growth factors, such as platelet rich plasma, bone marrow aspirate and biophysical stimulation could be adopted in order to improve biological stimulation (21-23). For example, biophysical stimulation is a non-surgical conservative and frequently used therapy in nonunions. A greater efficacy has been demonstrated for pulsed electromagnetic fields (PEMF), then for shock waves and ultrasounds (24, 25). PEMF showed good clinical results in the treatment of delayed unions and nonunions (8, 26-40).

Some meta-analyses are also available on this subject. Hannemann in 2014 showed how results from trials with substantial methodological quality indicated that PEMF decreases healing time (41). Mollon in 2008 found no significant effect of stimulation for nonunions (42). Griffin in 2011 concluded that the available evidence was inconclusive and insufficient to inform current practice (43). Moreover, in 2013, Behrens and colleagues found that clinical studies on PEMF for the treatment of nonunions were primarily studies of level IV (case series, retrospective studies without control group) (44). All these studies however indicate how it is difficult to determine the effectiveness of PEMF stimulation with the lack of adequately powered randomized controlled trials and the high levels of heterogeneity in methodology and utilized devices among studies.

As far as the *in vitro* studies on the effect of PEMF

is concerned, the mechanisms of action at cellular and molecular levels are still under debate. The group of Vincenzi found that PEMF improved the osteogenesis process by acting directly on osteoblasts (45, 46). Other authors demonstrated that PEMF stimulate the production of growth factors belonging the TGF-β/BMPs superfamily that could be responsible of the improvement in the osteogenetic process (47-49). Recently, it was reported that PEMF could also modulate osteogenic differentiation of bone marrow and amniotic membrane derived-mesenchymal stem cells (50-53).

However, all these studies dealt with healthy bone-derived cells. Only one study *in vitro* analyzed the effects and mechanism of action of PEMF on cells isolated from seven nonunion patients (54). Guerkov and colleagues found that PEMF did not influence cell proliferation, alkaline phosphatase, collagen, PGE_2 and osteocalcin deposition; on the contrary PEMF significantly enhanced the production of TGF-β1.

Finally, a great variability in the PEMF parameters, exposure times and length of duration was observed across both *in vitro* and clinical studies, varying from 0.1 to 20 G of electric field amplitude, from 3 to 20 h per day for a treatment duration ranging from 3 to 7 months (40).

Thus, the primary aim of the present research was to investigate the capacity of PEMF (with different exposure times: 4h, 8h and 16h/die) to stimulate the osteogenetic and vascular processes on human cells harvested from the callus of a nonunion patient. Cell morphology, proliferation, gene expression and protein analysis were performed at 7, 14 and 21 days of PEMF stimulation. The secondary aim was the assessment of the intrinsic characteristics of the cells isolated from the nonunion in terms of clonogenicity, cluster of differentiation expression and the tri-lineage differentiation capacity (adipogenic, chondrogenic and osteogenic), since only the group of Boyan indicated the presence of multipotent cells within canine and human nonunions (55,56).

MATERIALS AND METHODS

Patient and sample harvesting

A research protocol was designed for the submission to the Local Ethic Committee. Inclusion criteria were: patients aged between 18 and 50 years with a scheduled surgical treatment of reduction and stabilization of long bone nonunion (over 6 months after fracture) and able to give informed consent. Exclusion criteria were the presence of infective or neoplastic diseases, positive serology for HIV-HBV-HCV and pregnancy. Tissue from a hypertrophic nonunion was obtained after the signature of informed consent. During surgery, nonunion bone mocks were submitted to debridement: specimens of such tissues were aseptically harvested and placed in saline solution.

Cell isolation

Tissue samples were processed for cell isolation. Briefly, pieces were finely minced using a sterile scalpel, washed with Phosphate Buffered Saline (PBS) and cultured in 25 cm2 flasks in growth medium, containing Dulbecco's Modified Eagles Medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% Fetal Bovine Serum (FBS, Lonza, Verviers, Belgium), 100U/ml penicillin, 100μg/ml streptomycin (Gibco, Life Technologies, Carlsbad, CA, USA) and 5 μg/ml plasmocin (Invivogen, San Diego, CA, USA). Medium was replaced every two days until outgrowth cells reached confluence. Cells were then expanded to passage 2, when all experiments were performed.

Evaluation of cell immunophenotype by flow cytometry

Cells were detached and counted in a Neubauer chamber with Trypan Blue exclusion. A total amount of $5x10^5$ cells were used for antibody labeling. For each antigen, 10^5 cells were labeled with 5μl of the following antibodies: Fluorescein Isothiocyanate (FITC) anti-human CD73 (eBioscience, San Diego, CA, USA), FITC anti-human CD90 (Biolegend, San Diego, CA, USA), FITC anti-human CD105 (Biolegend), FITC anti-human CD45 (Biolegend) and FITC mouse IgG1, κ Isotype Control (Biolegend). The cells were analyzed with FACSCanto II cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) equipped with FACSDiva software (Becton Dickinson).

Colony Forming Unit – Fibroblast (CFU-F) assay

Two hundred cells were seeded in 25 cm^2 flasks in growth medium. After 14 days, cells were fixed with 10% neutral buffered formalin and stained with 0.1% Toluidin Blue. Colonies were then observed and counted in light microscopy and the percentage of colonies formed upon the initial number of cells were calculated.

Induction of tri-lineage differentiation

Cells were induced to tri-lineage differentiation in order to assess their plasticity.

Osteogenic differentiation: $2x10^4$ cells were seeded in 6-well plates in growth medium. After 24 h, medium

was replaced with an osteogenic medium (DIFF), i.e growth medium with the addition of 50 µg/ml ascorbate 2-phosphate, 7 mM β-glycerophosphate and 10^{-7}M dexamethasone (Sigma-Aldrich). Medium was then replaced every two days. Between 2 and 3 weeks of culture, some cells detached and structured themselves in a three-dimensional fashion: those masses at 21 days were then paraffin-embedded and stained with Stevenel's Blue and Picrofuchsin in order to evaluate the presence of an osteoid matrix. Analysis of the expression of osteogenic genes (*RUNX2*, *COL1A1*, *ALPL*, *SOX9, BGLAP, SPP1* and housekeeping genes) was performed in quantitative reverse transcription PCR (RT-qPCR), as described in a following section. Results were compared to undifferentiated cells grown in the same conditions but in growth medium (CRL).

Adipogenic differentiation: $2x10^4$ cells were seeded in 6-well plates in growth medium. After 24 h, medium was replaced with an adipogenic medium, i.e. growth medium with the addition of 10 µg/ml insulin, 500 µM isobutylmethylxanthine, 100 µM indomethacin, 1 µM dexamethasone. Medium was then replaced every two days. After 21 days, cultures were stained with Oil Red O to evaluate the presence of intracellular accumulation of lipids.

Chondrogenic differentiation: $2.5x10^5$ cells were centrifuged at 160g for 10 min in a 1.5 ml tube in order to form high-density micromasses. After three days, medium was replaced with a chondrogenic medium: Chondrogenic Basal Medium with supplements (Insulin-transferrin-sodium selenite, dexamethasone, ascorbate, sodium pyruvate, proline, GA-1000, L-glutamine) and 10 ng/ml TGF-β3 (Lonza). Medium was then replaced twice a week. After 28 days micromasses were paraffin-embedded and stained with Hematoxylin-Eosin and with Alcian Blue to evaluate the formation of a cartilaginous matrix.

Osteogenic differentiation and PEMF stimulation

Cells were seeded at a density of $1.5x10^4$ cells in 12-well plates for simultaneous induction of osteogenic differentiation and stimulation with PEMF. PEMF were delivered by a pulse generator (IGEA, Carpi, Italy) of weak intensity and low frequency (2.3 mT, 75Hz, 1.3 ms pulse duration): coils were applied perpendicularly to the multiwell plates and the selected exposure times were 4, 8 and 16 h per day.

The experimental groups were set as following:

- Differentiated control, i.e. cells grown in osteogenic medium (DIFF);
- Osteogenic medium + stimulation with PEMF, 4h/die (PEMF4);
- Osteogenic medium + stimulation with PEMF, 8h/die (PEMF8);
- Osteogenic medium + stimulation with PEMF, 16h/die (PEMF16).

At 7 and 14 days cells were tested for viability and proliferation, calcium deposition, immunoenzymatic assays (ELISA) and gene expression analyses. Between 2 and 3 weeks of culture, cells detached and structured themselves in a three-dimensional fashion: in this case only a histologic evaluation of masses could be performed at 21 days. Cells at T0 (before the beginning of stimulation) were used as a basal control for gene expression.

Cell viability

Cell viability was assessed by Alamar Blue assay. Briefly, cells were incubated with a solution of medium containing 10% of Alamar Blue reagent (AbD Serotec, Oxford, UK) for 3h30' at 37°C. Afterwards, the optical density of the supernatants was measured at 570 and 625 nm. The percentage of Alamar Blue reduction was calculated with the following formula:

$$[A_{LW} - (A_{HW} \times R_0)] \times 100$$

Where A_{LW} is the absorbance at 570 nm minus the media blank and A_{HW} is the absorbance at 625 nm minus the media blank. R_0 is a correction factor calculated as the ratio between the difference of the absorbance of Alamar Blue in media minus the absorbance of media only at the lower wavelength and the one at the higher wavelength.

Immunoenzymatic assays (ELISA)

At each time point, supernatants were centrifuged, aliquoted in 1.5 ml tubes and stored at -20°C until use.

Enzyme-linked immunosorbent assay (ELISA) kits were purchased from Boster Biological Technology (Pleasanton, CA, USA) and the concentration of the following secreted factors were measured following manufacturer's instruction: TGF-β1, MMP-1, MMP-7, MMP-8, MMP-12, TIMP-1, TIMP-2, BMP-2, BMP-4 and BMP-7. Data were normalized to the total RNA content and expressed as pg protein/ng total RNA.

Analysis of gene expression

Total RNA was extracted using the PureLink RNA Mini Kit (Life Technologies, Carlsbad, CA, USA) and reverse transcribed with the Superscript Vilo cDNA synthesis kit (Life Technologies), following the manufacturers' instructions. Complementary DNA (cDNA) was diluted to the final concentration of 5 ng/µl. Gene expression was evaluated by qPCR, using the SYBR green PCR kit (Qiagen, Hilden, Germany) in a Light Cycler 2.0 Instrument (Roche Diagnostics). Ten nanograms of each sample were tested in duplicate for the expression of

GAPDH, ACTB, PGK1, RUNX2, COL1A1, ALPL, BGLAP, SPP1, SOX9, VEGFA, TGFB1, BMP2, BMP4, BMP7, MMP1, MMP7, MMP8, MMP12, TIMP1 and TIMP2. Primer details are reported in Table I. The mean threshold cycle was used for the calculation of relative expression using the $2^{-\Delta\Delta Ct}$ method, using the Ct geometric mean of *GAPDH, ACTB and PGK1* as reference for calculation of ΔCt and using the control at T0 as the calibrator.

Analysis of formation of an osteoid matrix

After 21 days, cells that appeared as three dimensional structures were fixed in 10% neutral buffered formalin solution, extensively rinsed in distilled water, dehydrated in graded alcohol solutions (70%, 95% and 100%), cleared in xylene e finally paraffin embedded. Sections (5±1 µm) were cut by a semi-automated microtome (Microm H340E, Germany) and stained with Stevenel's Blue and Picrofuchsin in order to evaluate the presence of osteoid matrix.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (v.6.0). Unpaired t-tests were performed to compare means between undifferentiated (CRL) and differentiated (DIFF) samples. To evaluate the differences among treatments (DIFF, PEMF4, PEMF8, PEMF16), one-way ANOVA tests followed by Holm-Sidak multiple comparisons were used. P-value was set at 0.05.

RESULTS

Characterization of nonunion cells

At the second passage, nonunion cells were characterized in order to evaluate the presence of a progenitor population. Flow cytometry analysis showed that the cells were negative for CD45 and almost 100% positive for CD73, CD90 and CD105 (Fig. 1).

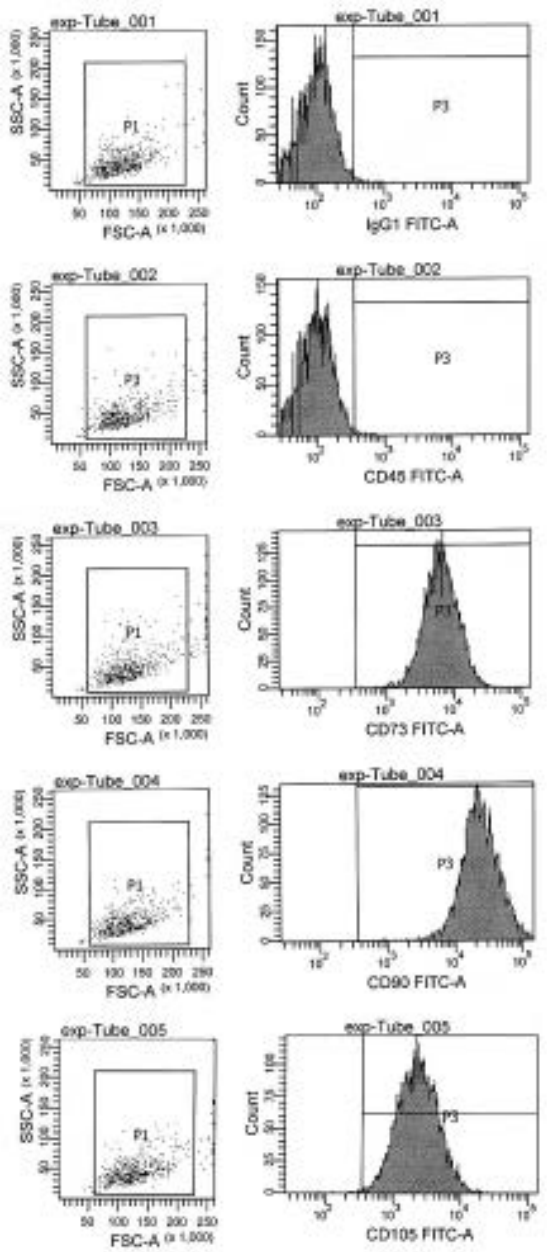


Fig. 1. *Flow cytometry characterization of nonunion cells immunophenotype.*

The clonogenic ability of nonunion cells was tested with the CFU-F assay. After 14 days of culture, 29 colonies were counted, thus the percentage of colony forming units on the number of seeded cells was 14.5%. (Fig. 2A).

In order to assess plasticity, nonunion cells were stimulated towards osteogenic, chondrogenic and adipogenic differentiation. The results showed that the cells were partially addressed towards a osteogenic lineage: at 21 days the cells moderately stained with Alizarin Red dye (Fig. 2B-C). The cells stimulated towards a chondrogenic differentiation showed a diffuse staining with Alcian Blue, demonstrating the formation of a cartilaginous matrix composed by glycosaminoglycans (Fig. 2D-E). Conversely, the cells did not show signs of adipogenic differentiation by Oil Red O staining (Fig. 2F-G).

The process of osteogenic differentiation was

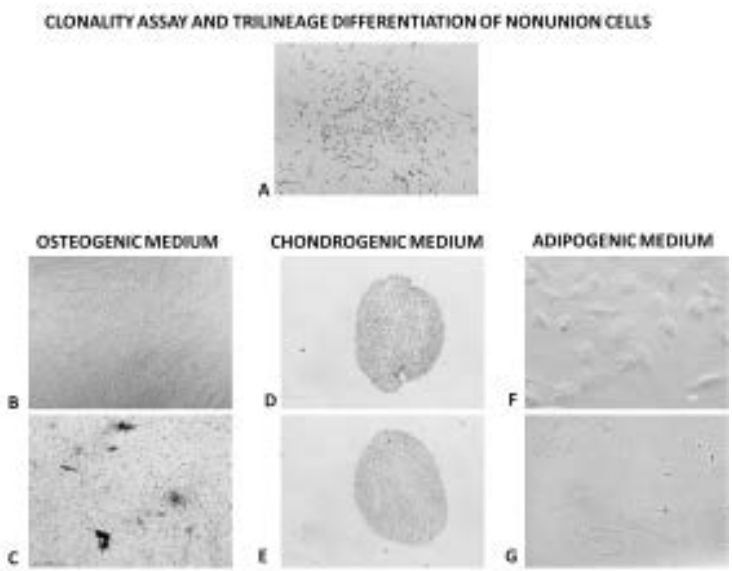


Fig. 2. *Clonality assay (**A**) and tri-lineage differentiation (**B-G**) of human cells harvested from the nonunion. **A**): Cells at the second passage were seeded in basal medium and, after 14 days of culture, stained with Toluidine Blue to stain and count the Colony Forming Units. Magnification 4X. Bar = 500μm; **B-C**): Cells at the second passage were cultured in osteogenic differentiation medium for 21 days. Images by light optic microscope were captured on unstained (**B**) or after staining with Alizarin Red to identify mineralization foci (**C**). Magnification 4X. Bar = 500 μm; **D-E**): Cells at the second passage were seeded in a micromass culture condition and in chondrogenic differentiation medium for 28 days. Sections of micromasses were stained with Harris Haematoxilin-Eosin Y (**D**) and with Alcian Blue (**E**) to evaluate morphology and glycosaminoglycan deposition, respectively. Magnification 20X. Bar = 100 μm; **F-G**): Cells at the second passage were cultured in adipogenic medium for 21 days. Images by light optic microscope were captured on unstained (**F**) or after staining with Oil Red to identify lipid vacuoli (**G**). Magnification 20X. Bar = 100 μm.*

examined more in depth by comparing the gene expression analysis of nonunion cells cultured in osteogenic medium (DIFF) or in basal medium as control (CRL). The results showed that *RUNX2* expression increased significantly ($p<-0.01$) in osteogenic medium in comparison with CRL after 3 weeks from induction (Fig. 3A). *COL1A1* was downregulated at 2 weeks ($p<0.05$); its expression increased again at 3 weeks, consistently with the expression of *RUNX2*, although without reaching a statistical significance (Fig. 3B). *ALPL* was strongly upregulated at all time points ($p<0.01$ at 1 and 2 weeks and $p<0.001$ at 3 weeks) (Fig. 3C). Finally, the expression of two late osteogenic markers, *BGLAP* (osteocalcin) and *SPP1* (osteopontin) was not evaluable in any sample (data not shown).

The expression of *SOX9* was also evaluated (Fig. 3D). The analysis showed a slight decrease in osteogenic medium in comparison with CRL during differentiation at 1 week ($p<0.05$).

Effect of PEMF on cell proliferation

The treatment with PEMF for 4h/day or 16h/day improved cell viability at 1 week, as illustrated by the increase in the percentage of Alamar Blue reduction of 16.36% ($p<0.01$) and 23.91% ($p<0.0001$), respectively (Fig. 4**)** in comparison with DIFF.

Effect of PEMF on osteogenic differentiation of nonunion cells

At time points after 2 weeks, the cells started to detach as the result of a hyperconfluent state. In this context, the tridimensional structures that formed were paraffin-embedded and stained with Stevenel's Blue and Picrofuchsin in order to evaluate the presence of an osteoid matrix. The results showed

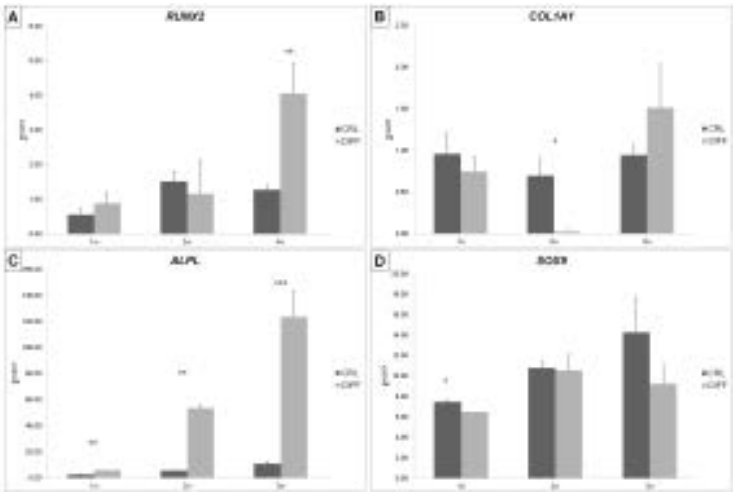


Fig. 3. *Expression levels of osteogenic markers and SOX9 during differentiation. (**A**): RUNX2; (**B**): COL1A1; (**C**): ALPL; (**D**): SOX9. **CRL**: undifferentiated cells; **DIFF**: cells undergoing osteogenic differentiation. *: p<0.05; **: p<0.01; ***: p<0.001.*

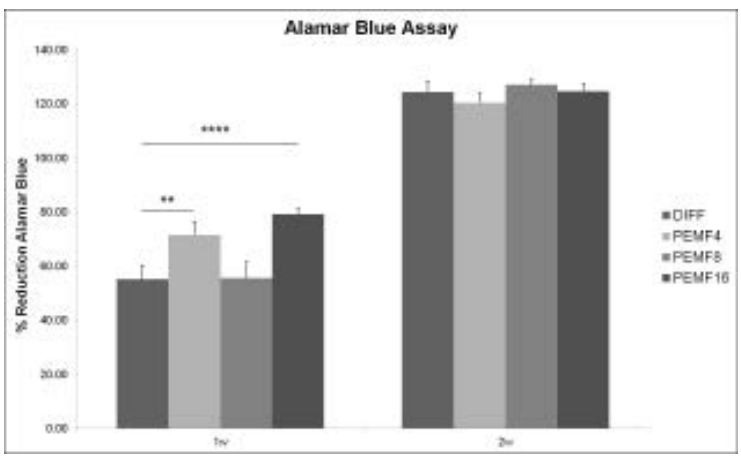


Fig. 4. *Cell viability analysis with Alamar Blue assay. **: p<0.01; ****: p<0.0001.*

osteoid formation which increased proportionally to PEMF exposure, being more evident in PEMF16 (Fig. 5). In all samples, however, the staining was not very strong, indicating that cells did not reach late stages of differentiation.

Gene expression analysis indicated that *RUNX2* levels increased significantly over DIFF at 1 weeks in PEMF8 and PEMF16 ($p<0.05$, Fig. 6A). The expression of *COL1A1* was upregulated at 2 weeks in the same treated samples ($p<0.01$ and $p<0.05$, respectively; Fig. 6B). The expression of *ALPL* showed no differences among treatments (Fig. 6C). The expression of late osteogenic markers *BGLAP* and *SPP1* was still unassessable. The expression of *SOX9* decreased when cells were treated with PEMF in comparison with DIFF, regardless of the exposure (PEMF4, $p<0.05$; PEMF8 and 16, $p<0.01$; Fig. 6D).

Effect of PEMF on the expression of growth factors

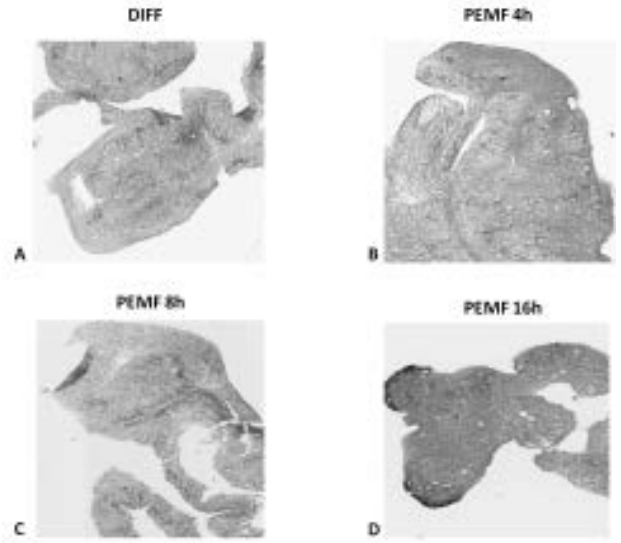


Fig. 5. *Histological images of cells that after 21 day in culture formed 3D structures. Newly deposited osteoid matrix stains green.* ***A****): Cells grown in osteogenic medium;* ***B****): Cells grown in osteogenic medium and stimulated with PEMF 4h/die;* ***C****): Cells grown in osteogenic medium and stimulated with PEMF 8h/die;* ***D****): Cells grown in osteogenic medium and stimulated with PEMF 16h/die. Magnification 10X. Stevenel Blue and Picrofuchsin staining.*

The results failed to point out significant differences between treatments for BMP-2, BMP-7 and TGF-β1 (data not shown). *VEGFA*, assayed in RT-qPCR, showed a significant increase in PEMF16, in comparison with DIFF, at 1 week ($p<0.05$, Fig. 7A).

Noteworthy, BMP-4 protein levels showed a peak in PEMF8 at 1 week (87.2±4 pg BMP-4/ng total RNA) (Fig. 7B).

Effect of PEMF on the expression of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs)

The expression of MMP-1, MMP-7, MMP-8 and MMP-12 did not differ significantly between treatments.

The levels of MMP inhibitors, *TIMP1* and *TIMP2*, showed slight variations at the levels of gene expression. In particular, *TIMP1* expression was increased in PEMF4 at two weeks ($p<0.05$, Fig. 7C) in comparison with DIFF, whereas *TIMP2* levels were downregulated in PEMF8 and PEMF16 at 2 weeks ($p<0.05$, Fig. 7D). No differences were found in TIMPs protein levels.

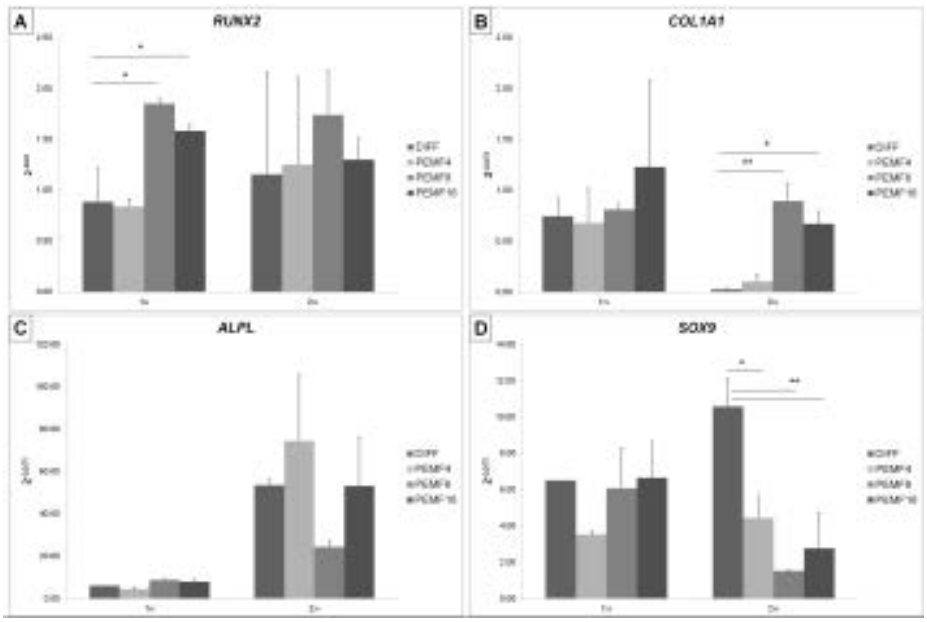


Fig. 6. *Expression levels of osteogenic markers and SOX9 during differentiation and PEMF stimulation.* ***A****): RUNX2;* ***B****): COL1A1, (****C****) ALPL, (****D****) SOX9; *: $p<0.05$; **: $p<0.01$.*

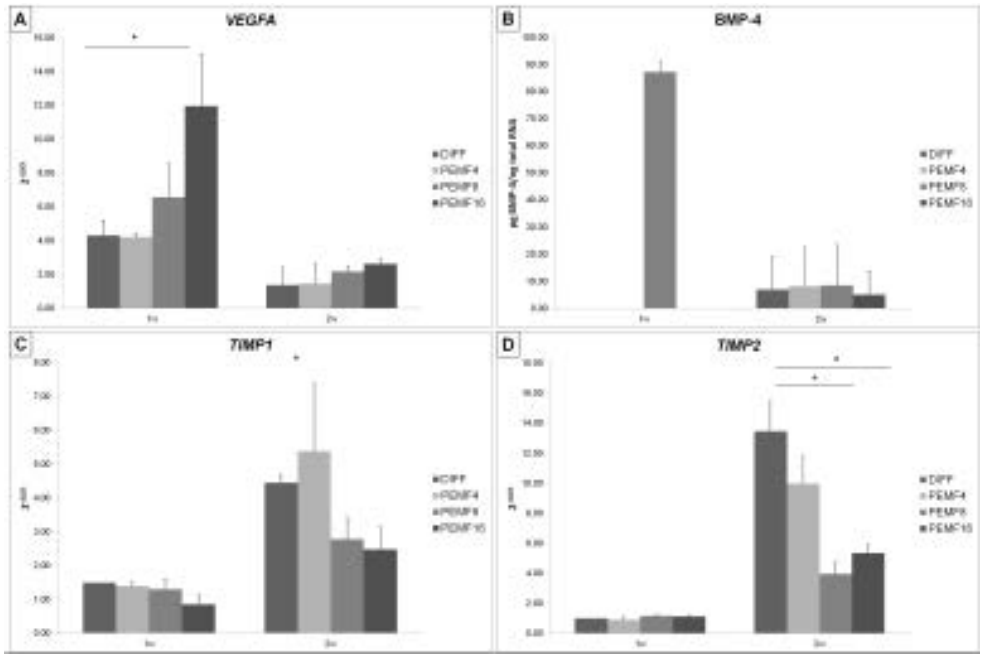


Fig. 7. *Significative results on growth factors and TIMPs expression after stimulation with PEMF. **A**): VEGFA, gene expression; **B**): BMP-4, protein levels; **C**): TIMP1, gene expression; **D**): TIMP2, gene expression; *****: p<0.05.*

Table I. *Primers used for gene expression analysis.*

Gene	Primer Forward (5'→3')	Primer Reverse (5'→3')	Annealing	Notes
GAPDH	TGGTATCGTGGAAGGACTCA	GCAGGGATGATGTTCTGGA	56°C	
ACTB	CCTTGCACATGCCGGAG	ACAGAGCCTCGCCTTTG	60°C	PrimeTime qPCR primers, IDT
PGK1	CTAACAAGCTGACGCTGGA	GACAGCAGCCTTAATCCTCTG	60°C	PrimeTime qPCR primers, IDT
RUNX2	CTTCACAAATCCTCCCCAAGT	AGGCGGTCAGAGAACAAAC	60°C	PrimeTime qPCR primers, IDT
COL1A1	QuantiTect Primer Assay Hs_COL1A1_1_SG		55°C	Qiagen
ALPL	QuantiTect Primer Assay (Qiagen) Hs_ALPL_1_SG		55°C	Qiagen
BGLAP	ACACTCCTCGCCCTATTG	GATGTGGTCAGCCAACTC	55°C	
SPP1	QuantiTect Primer Assay Hs_SPP1_1_SG		55°C	Qiagen
SOX9	GAGCAGACGCACATCTC	CCTGGGATTGCCCCGA	60°C	
VEGFA	QuantiTect Primer Assay Hs_VEGFA_6_SG		55°C	Qiagen
TGFB1	GTTCAGGTACCGCTTCTCG	CCGACTACTACGCCAAGGA	60°C	PrimeTime qPCR primers, IDT
BMP2	TTTGACCAGAGTTTTTCCATG	GAAGCAGCAACGCTAGAAGA	60°C	PrimeTime qPCR primers, IDT
BMP4	GAGCCTTTCCAGCAAGTTTG	CCATCAGCATTCGGTTACCA	60°C	PrimeTime qPCR primers, IDT
BMP7	CAGCCTGCAAGATAGCCATT	ATCAAACCGGAACTCTCGATG	60°C	PrimeTime qPCR primers, IDT
MMP1	GACAGAGATGAAGTCCGGTTT	GCCAAAGGAGCTGTAGATGTC	60°C	PrimeTime qPCR primers, IDT
MMP7	GATGAGGATGAACGCTGGA	GAATGTCCCATACCCAAAGAATG	60°C	PrimeTime qPCR primers, IDT
MMP8	GCTTCCATTTCTGCTCTTACTC	GCCATTCTTCCTTGTAGACTGA	60°C	PrimeTime qPCR primers, IDT
MMP12	CTCTCTGCTGATGACATACGTG	CAAAACTCAAATTGGGGTCACAG	60°C	PrimeTime qPCR primers, IDT
TIMP1	CCGACCTCGTCATCAG	GTTGTGGGACCTGTGGAA	60°C	PrimeTime qPCR primers, IDT
TIMP2	GACGTTGGAGGAAAGAAGGA	TGTGGTTCAGGCTCTTCTTC	60°C	PrimeTime qPCR primers, IDT

DISCUSSION

Although PEMF are beneficial and already used in the treatment of nonunions, the exact mechanism of action on cells and tissues in normal healing and nonunion is still unclear. This could be explained by two main reasons. The first is the lack of studies *in vitro* on cells and tissues isolated from the pathological sites, combined with the paucity of data on dose-response effects. Moreover, it is still difficult to understand the myriad of biological alterations implied and which ones have a primary role in the development of a nonunion. This study presents some preliminary results and it is a part of a larger project that aims to fill some of these gaps.

One of the purposes was to isolate and characterize cells from the callus of nonunion patients. The cells were isolated without enzymatic digestion of the tissue in order to preserve the native characteristics of cells and microenvironment as far as possible.

The cultures at passage two were heterogeneous, with some differences in cell morphology (dimension and shape) observed at light microscopy. Interestingly, the cells were homogeneous in the expression of typical mesenchymal markers such as CD73, CD90 and CD105 and were negative for CD45, indicating at least a common mesenchymal origin.

In those cultures, a stem/progenitor population was accounting for at least the 14.5%, as indicated from the results of CFU-F assay. In addition, cells were induced to classic trilineage differentiation. While the cells could not tri-lineage into adipocytes, the results showed that nonunion cells were able to differentiate towards a chondrogenic and an osteogenic lineage, at least in part. Accordingly, the analysis of gene expression during osteogenic differentiation showed that there was a late activation of *RUNX2*, the master transcription factor regulating the expression of downstream markers of osteogenesis and a simultaneous downregulation of *SOX9*. The expression of *COL1A1*, encoding for the chain α1 of collagen type I, also increased at late time points. These results seem to point out that the differentiation process is delayed in cells directly derived from pathological callus, starting at three weeks. At this time, normal human mesenchymal stem cells in the same culture conditions usually show the expression of such genes before and at three weeks of culture they already show late osteogenetic differentiation and mineralization. Thus, in our study, nonunion cells showed an impairment in their differentiation ability. This should not be surprising considering the pathological tissue of origin.

Overall, these preliminary data indicate the presence of a multipotent population in the callus of a nonunion, probably osteochondroprogenitor cells. To the authors' knowledge there are no studies, except that of the group of Boyan (55) demonstrating the presence of stem/progenitor cells in the site of a nonunion. The paper by Mathieu et al. (16) reported a reduced pool and proliferation capacity of bone marrow mesenchymal stem cells, however isolated from the iliac crest and not from the callus.

Afterwards, the main question to be addressed was to evaluate the effect of PEMF on nonunion cells, in terms of viability, differentiation and expression of some of the factors that were reported to be altered in nonunions (10, 12, 15, 17, 18, 54). Moreover, an initial evaluation of a dose-response curve with PEMF was performed, by utilizing different exposure times per day (4, 8 and 16 h) with a PEMF generator commercially available and with the same parameters (2.3 mT, 75Hz, 1.3 ms pulse duration) as those already utilized in clinics to enhance bone repair and fracture healing (57).

The results showed that PEMF may enhance cell viability, induce a higher formation of osteoid matrix, as indicated from histology and accelerate the process of osteogenic differentiation, as indicated from gene expression analysis of *RUNX2* and *COL1A1*. Moreover, we observed an early and higher expression of BMP-4 in cells treated with PEMF for 8h/day, an upregulation of *TIMP1* in PEMF 4h/day and a downregulation of *TIMP2* in cells exposed for 4 and 8h/day. *VEGFA* upregulation, observed in PEMF16h/day at 1 week, may account for a possible improvement in both the osteogenic and vasculogenic processes.

Data available from literature indicate that PEMF can stimulate osteogenesis in several cell types, including human bone marrow and adipose tissue derived-mesenchymal stem cells (58), human alveolar bone-derived mesenchymal stem cells (59), amniotic epithelial cells (53) and SaOS-2 cell line (60). However, all these studied dealt with

healthy cells. Only Guerkov (54) studied the effect of PEMF on cells derived from nonunion tissues. The authors observed no differences in cell number, proliferation, alkaline phosphatase activity and osteocalcin, collagen and PGE_2 production. They observed a higher production of TGF-β1 in PEMF-treated cultures. However, in that study, cells were stimulated 8h/day for 4 days, so it is very difficult to compare our results.

The main limitation of the present study is that it reports some preliminary results of experiments conducted on samples derived from a single patient and not having cells from a normal healing tissue, as a control. While interesting to address the future research, it is clear that it is not sufficient to draw definitive conclusions. Moreover, regarding PEMF exposure, not only the exposure times/day could be varied, but also other parameters, such as the intensity and frequency of the electromagnetic fields.

Another limitation was that evaluations were mostly limited at 2 weeks of culture. Indeed, between 2 and 3 weeks, cells detached and formed three-dimensional structures. Thus, only histologic analyses were performed because other evaluations would be compromised by this alteration in cultures. If PEMF stimulate cell viability and proliferation, a solution may be represented by reducing initial cell density. Similarly, a review focusing on the use of low level laser therapy (LLLT), known to stimulate the proliferation of several cell types, suggests that cultures should be 20% confluent at the time of exposure to obtain the maximum yield from LLLT (61). This suggestion should be translated to the use of PEMF.

Despite those drawbacks, this study is to our knowledge one the first to report the presence of a progenitor population, accordingly to the minimal criteria (62), in the site of a nonunion and to deeply investigate their osteogenic commitment in a human subject. The isolation technique employed, which does not comprise a step of enzymatic digestion, on one hand results in a more heterogeneous culture, but it better preserves the characteristics of cells and microenvironment of the native tissue. Another strong point was studying the effect of three different exposure times of PEMF on cells derived from a pathological site, for a three-week time span.

In conclusion, even with the limitations above discussed, these findings suggest the presence of a multipotent progenitor cell population in the fracture callus of nonunion patients. These cells, if exposed to PEMF, increase viability, osteogenic commitment and bone matrix deposition. Moreover, from these preliminary data, that have to be confirmed by broad investigations on many other patients, it seems that the response is dose-dependent with longer exposures/day inducing better effects both on anabolic properties of cells and on growth factor patterns. Furthermore, if those findings will be confirmed, it may open the possibility of testing PEMF on other diseases characterized by uneventful bone healing, such as peri-implant osteolysis.

ACKNOWLEDGEMENTS

The study was supported by "Programma di Ricerca Regione Emilia-Romagna-Università 2010-2012" to MT (Biological and Biophysical Stimulation of Implant Osteolysis and Aseptic Loosening Conditions: Effects of Pulsed Electromagnetic Fields and Platelet Derivatives Project).

The authors wish to thank IGEA S.p.A. for providing PEMF generator and Luca Cattini for flow cytometer analyses.

REFERENCES

1. Harwood PJ, Newman JB, Michael ALR. An update on fracture healing and non-union. Orthopaedics and Trauma 2010; 24(1):9-23.
2. Hak DJ, Fitzpatrick D, Bishop JA, Marsh JL, Tilp S, Schnettler R, Simpson H, Alt V. Delayed union and nonunions: epidemiology, clinical issues and financial aspects. Injury 2014; 45(S)2:3-7.
3. Frost HM. The biology of fracture healing: an overview for clinicians. Part I. Clin Orthop Rel Res 1989; 248:283-93.
4. Karadimas EJ, Papadimitriou G, Theodoratos G, Papanikolaou A, Maris J. The effectiveness of the antegrade reamed technique: the experience and complications from 415 traumatic femoral shaft fractures. Strat Traum Limb Recon 2009; 4:113-21.
5. Kovar FM, Jaindl M, Schuster R, Endler G, Platzer P. Incidence and analysis of open fractures of the midshaft and distal femur. Wien Klin Wochenschr

2013; 125:396-401.
6. Phieffer LS, Goulet JA. Delayed unions of the tibia. J Bone Joint Surg 2006; 88A:206-16.
7. Mariconda M, Cozzolino F, Cozzolino A, D'Agostino E, Bove A, Milano C. Platelet gel supplementation in long bone nonunions treated by external fixation. J Orthop Trauma 2008; 22(5):342-5.
8. Shi HF, Xiong J, Chen YX, Wang JF, Qiu XS, Wang YH, Qiu Y. Early application of pulsed electromagnetic field in the treatment of postoperative delayed union of long-bone fractures: a prospective randomized controlled study. BMC Musculoskeletal Dis 2013; 14:35.
9. Frost HM. The biology of fracture healing: an overview for clinicians. Part II. Clin Orthop Rel Res 1989; 248:294-309.
10. Zimmermann G, Henle P, Küsswetter M, Moghaddam A, Wentzensen A, Richter W, Weiss S. TGF-beta1 as a marker of delayed fracture healing. Bone 2005; 36(5):779-85.
11. Zimmermann G, Schmeckenbecher KH, Boeuf S, Weiss S, Bock R, Moghaddam A, Richter W. Differential gene expression analysis in fracture callus of patients with regular and failed bone healing. Injury 2012; 43(3):347-56.
12. Fajardo M, Liu CJ, Egol K. Levels of expression for BMP-7 and several BMP antagonists may play an integral role in a fracture nonunion: a pilot study. Clin Orthop Relat Res 2009; 467(12):3071-8.
13. Weiss S, Zimmermann G, Pufe T, Varoga D, Henle P. The systemic angiogenic response during bone healing. Arch Orthop Trauma Surg 2009; 129(7):989-97.
14. Weiss S, Henle P, Bidlingmaier M, Moghaddam A, Kasten P, Zimmermann G. Systemic response of the GH/IGF-I axis in timely versus delayed fracture healing. Growth Horm IGF Res 2008; 18(3):205-12.
15. Sarahrudi K, Thomas A, Braunsteiner T, Wolf H, Vécsei V, Aharinejad S. VEGF serum concentrations in patients with long bone fractures: a comparison between impaired and normal fracture healing. J Orthop Res 2009; 27(10):1293-7.
16. Mathieu M, Rigutto S, Ingels A, et al. Decreased pool of mesenchymal stem cells is associated with altered chemokines serum levels in atrophic nonunion fractures. Bone 2013; 53(2):391-8.
17. Fajardo M, Liu CJ, Ilalov K, Egol KA. Matrix metalloproteinases that associate with and cleave bone morphogenetic protein-2 in vitro are elevated in hypertrophic fracture nonunion tissue. J Orthop Trauma 2010; 24(9):557-63.
18. Henle P, Zimmermann G, Weiss S. Matrix metalloproteinases and failed fracture healing. Bone 2005; 37(6):791-8.
19. Calori GM, Capanna R, Colombo M, De Biase P, O'Sullivan C, Cartareggia V, Conti C. Cost effectiveness of tibial nonunion treatment: A comparison between rhBMP-7 and autologous bone graft in two Italian centres. Injury 2013; 44(12):1871-9.
20. LaVelle DG, Canale ST, Beaty JH. Campbell's Operative Orthopedics. Philadelphia, Pennsylvania: Mosby Inc 2003; 3228-9.
21. Campana V, Milano G, Pagano E, Barba M, Cicione C, Salonna G, Lattanzi W, Logroscino G. Bone substitutes in orthopaedic surgery: from basic science to clinical practice. J Mater Sci Mater Med 2014; 25(10):2445-61.
22. Malhotra R, Kumar V, Garg B, Singh R, Jain V, Coshic P, Chatterjee K. Role of autologous platelet-rich plasma in treatment of long-bone nonunions: a prospective study. Musculoskelet Surg 2015; (Epub ahead of print).
23. Virk MS, Lieberman JR. Biologic adjuvants for fracture healing. Arthritis Res Ther 2012; 14(6):225.
24. Elster EA, Stojadinovic A, Forsberg J, Shawen S, Andersen RC, Schaden W. Extracorporeal shock wave therapy for nonunion of the tibia. J Orthop Trauma 2010; 24(3):133-41.
25. Hannemann P, Göttgens KW, van Wely BJ, Kolkman KA, Werre AJ, Poeze M, Brink PR. Pulsed Electromagnetic Fields in the treatment of fresh scaphoid fractures. A multicenter, prospective, double blind, placebo controlled, randomized trial. BMC Musculoskelet Disord 2011; 12:90.
26. Heckman JD, Ingram AJ, Loyd RD, Luck JV Jr, Mayer PW. Nonunion treatment with pulsed electromagnetic fields. Clin Orthop Relat Res 1981; 161:58-66.
27. Barker AT, Dixon RA, Sharrard WJ, Sutcliffe ML. Pulsed magnetic field therapy for tibial non-union. Interim results of a double-blind trial. Lancet 1984;

1(8384):994-6.
28. Hinsenkamp M, Ryaby J, Burny F. Treatment of non-union by pulsing electromagnetic field: European multicenter study of 308 cases. Reconstr Surg Traumatol 1985; 19:147-51.
29. Borsalino G, Bagnacani M, Bettati E, et al. Electrical stimulation of human femoral intertrochanteric osteotomies. Double-blind study. Clin Orthop Relat Res 1988; 237:256-63.
30. Colson DJ, Browett JP, Fiddian NJ, Watson B. Treatment of delayed- and non-union of fractures using pulsed electromagnetic fields. J Biomed Eng 1988; 10(4):301-4.
31. Meskens MW, Stuyck JA, Feys H, Mulier JC. Treatment of nonunion using pulsed electromagnetic fields: a retrospective follow-up study. Acta Orthop Belg 1990; 56(2):483-8.
32. Sharrard WJ. A double-blind trial of pulsed electromagnetic fields for delayed union of tibial fractures. J Bone Joint Surg Br 1990; 72(3):347-55.
33. Garland DE, Moses B, Salyer W. Long-term follow-up of fracture nonunions treated with PEMFs. Contemp Orthop 1991; 22(3):295-302.
34. Traina GC, Fontanesi G, Costa P, Mammi GI, Pisano F, Giancecchi F, Adravanti P. Effect of electromagnetic stimulation on patients suffering from non-union. A retrospective study with a control group. J Bioelectricity 1991; 10:101-17.
35. Adams BD, Frykman GK, Taleisnik J. Treatment of scaphoid nonunion with casting and pulsed electromagnetic fields: a study continuation. J Hand Surg Am 1992; 17(5):910-4.
36. Gossling HR, Bernstein RA, Abbott J. Treatment of ununited tibial fractures: a comparison of surgery and pulsed electromagnetic fields (PEMF). Orthopedics 1992; 15(6):711-9.
37. Holmes GB Jr. Treatment of delayed unions and nonunions of the proximal fifth metatarsal with pulsed electromagnetic fields. Foot Ankle Int 1994; 15(10):552-6.
38. Simonis RB, Parnell EJ, Ray PS, Peacock JL. Electrical treatment of tibial non-union: a prospective, randomised, double-blind trial. Injury 2003; 34:357-62.
39. Cebrián JL, Gallego P, Francés A, Sánchez P, Manrique E, Marco F, López-Durán L. Comparative study of the use of electromagnetic fields in patients with pseudoarthrosis of tibia treated by intramedullary nailing. Int Orthop 2010; 34(3):437-40.
40. Assiotis A, Sachinis NP, Chalidis BE. Pulsed electromagnetic fields for the treatment of tibial delayed unions and nonunions. A prospective clinical study and review of the literature. J Orthop Surg Res 2012; 7:24.
41. Hannemann PF, Mommers EH, Schots JP, Brink PR, Poeze M. The effects of low-intensity pulsed ultrasound and pulsed electromagnetic fields bone growth stimulation in acute fractures: a systematic review and meta-analysis of randomized controlled trials. Arch Orthop Trauma Surg 2014; 134(8):1093-106.
42. Mollon B, da Silva V, Busse JW, Einhorn TA, Bhandari M. Electrical stimulation for long-bone fracture-healing: a meta-analysis of randomized controlled trials. J Bone Joint Surg Am 2008; 90(11):2322-30.
43. Griffin XL, Costa ML, Parsons N, Smith N. Electromagnetic field stimulation for treating delayed union or non-union of long bone fractures in adults. Cochrane Database Syst Rev 2011; (4):CD008471.
44. Behrens SB, Deren ME, Monchik KO. A Review of Bone Growth Stimulation for Fracture Treatment. Curr Orthop Pract 2013; 24(1):84-91.
45. Vincenzi F, Targa M, Corciulo C, et al. Pulsed electromagnetic fields increased the anti-inflammatory effect of A2A and A3 adenosine receptors in human T/C-28a2 chondrocytes and hFOB 1.19 osteoblasts. PLoS One 2013; 8(5):e65561.
46. Vincenzi F, Papalia R, Ruzzini L, Sgambato A, Maffulli N, Denaro V. Effect of pulsed electromagnetic fields on human osteoblast cultures. Physiother Res Int 2013; 18(2):109-14.
47. Schwartz Z, Simon BJ, Duran MA, Barabino G, Chaudhri R, Boyan BD. Pulsed electromagnetic fields enhance BMP- 2 dependent osteoblastic differentiation of human mesenchymal stem cells. J Orthop Res 2008; 26:1250-5.
48. Sollazzo V, Scapoli L, Palmieri A, Fanali S, Girardi A, Farinella F, Pezzetti F, Carinci F. Early effects of pulsed electromagnetic fields on human osteoblasts and mesenchymal stem cells. Europ J Infl 2011;

1(S):95-10.
49. Esposito M, Lucariello A, Riccio I, Riccio V, Esposito V, Riccardi G. Differentiation of human osteoprogenitor cells increases after treatment with pulsed electromagnetic fields. In Vivo 2012; 26:299-304.
50. Ceccarelli G, Bloise N, Mantelli M, et al. A Comparative analysis of the in vitro effects of pulsed electromagnetic field treatment on osteogenic differentiation of two different mesenchymal cell lineages. Bio Res Open Access 2013; 2(4):283-94.
51. Song MY, Yu JZ, Zhao DM, et al. The time-dependent manner of sinusoidal electromagnetic fields on rat bone marrow mesenchymal stem cells proliferation, differentiation and mineralization. Cell Biochem Biophys 2014; 69(1):47-54.
52. Sun LY, Hsieh DK, Lin PC, Chiu HT, Chiou TW. Pulsed electromagnetic fields accelerate proliferation and osteogenic gene expression in human bone marrow mesenchymal stem cells during osteogenic differentiation. Bioelectromagnetics 2010; 31(3):209-19.
53. Wang Q, Wu W, Han X, et al. Osteogenic differentiation of amniotic epithelial cells: synergism of pulsed electromagnetic field and biochemical stimuli. BMC Musculoskeletal Disorders 2014; 15:271.
54. Guerkov HH, Lohmann CH, Liu Y, Dean DD, Simon BJ, Heckman JD, Schwartz Z, Boyan BD. Pulsed electromagnetic fields increase growth factor release by nonunion cells. Clin Orthop Relat Res 2001; (384):265-79.
55. Boyan BD, Schwartz Z, Swain LD, Khare AG, Heckman JD, Ramirez V, Peters P, Carnes DL Jr. Initial effects of partially purified bone morphogenetic protein on the expression of glycosaminoglycan, collagen and alkaline phosphatase in nonunion cell cultures. Clin Orthop Relat Res 1992; 278:286-304.
56. Boyan BD, Caplan AI, Heckman JD, Lennon DP, Ehler W, Schwartz Z. Osteochondral progenitor cells in acute and chronic canine nonunions. J Orthop Res 1999; 17(2):246-55.
57. Aaron RK, Bolander ME. Physical Regulation of Skeletal Repair. Rosemont, Illinois. American Academy of Orthopaedic Surgeons 2005.
58. Ongaro A, Pellati A, Bagheri L, Fortini C, Setti S, De Mattei M. Pulsed electromagnetic fields stimulate osteogenic differentiation in human bone marrow and adipose tissue derived mesenchymal stem cells. Bioelectromagnetics 2014; 35(6):426-36.
59. Lim K, Hexiu J, Kim J, Seonwoo H, Cho WJ, Choung PH, Chung JH. Effects of electromagnetic fields on osteogenesis of human alveolar bone-derived mesenchymal stem cells. Biomed Res Int 2013; 2013:296019.
60. Martino CF, Belchenko D, Ferguson V, Nielsen-Preiss S, Qi HJ. The effects of pulsed electromagnetic fields on the cellular activity of SaOS-2 cells. Bioelectromagnetics 2008; 29(2):125-32.
61. AlGhamdi KM, Kumar A, Moussa NA. Low-level laser therapy: a useful technique for enhancing the proliferation of various cultured cells. Lasers Med Sci. 2012 Jan;27(1):237-49.
62. Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006; 8(4):315-7.

CELLULAR MECHANISMS OF BONE REGENERATION: ROLE OF WNT-1 IN BONE-MUSCLE INTERACTION DURING PHYSICAL ACTIVITY

G. COLAIANNI, C. CUSCITO, T. MONGELLI, P. PIGNATARO, R. TAMMA, A. ORANGER, S. COLUCCI AND M. GRANO

Department of Basic Medical Science, Neuroscience and Sense Organs. Section of Human Anatomy and Histology, University of Bari, 70124 Bari, Italy

Wnt1 is one of the several glycoproteins activating Wnt signaling, critical for normal skeletal development and bone homeostasis. Wnt1 was previously believed to solely regulate central nervous system development, in particular in midbrain and cerebellum. However, remarkable findings have recently shown that several patients affected by severe form of Osteogenesis Imperfecta (OI) display a Wnt1 mutation thereby revealing a possible role of Wnt1 in bone metabolism. Here, we show that recombinant Wnt1 (r-Wnt1) strongly increases differentiation of bone marrow stromal cells into mature osteoblasts, as demonstrated by the enhanced number of cells positively stained for alkaline phosphatase, one of the osteoblastic marker genes, whose mRNA levels are also significantly up-regulated. Furthermore, other osteogenic master genes such as Collagen I and Osteopontin are also enhanced when bone marrow precursors were differentiated toward osteoblastic phenotype in the presence of r-Wnt1. Intriguingly, by *in vivo* and *in vitro* findings, we report that in the bone marrow of mice subjected to physical activity there is a high endogenous Wnt1 synthesis compared to mice kept in resting conditions. Moreover, conditioned medium collected from *ex vivo* myoblasts, harvested from exercised mice, up-regulates Wnt1 expression in osteoblast cell cultures obtained from control mice. Overall our findings support the role of Wnt1 in regulating bone metabolism and suggest that this molecule could be one of the mediators through which physical activity may exert beneficial effect on bone.

Growing evidences indicate that Wnt signaling is a key regulator of bone homeostasis and crucial for skeletal development (1). At present, in both humans and mice, researchers have identified 19 different Wnt proteins which are specific to certain cells and tissues. Wnt proteins activate intracellular signals by binding to transmembrane receptor proteins, belonging to Frizzled family, and co-receptors, including low density lipoprotein receptor-related protein (LRP) 5 as well as LRP6, Ror2, and Ryk. At least three different Wnt intracellular signals are known: the Wnt/β-catenin pathway (also called canonical Wnt pathway), the non-canonical Wnt pathway, and the Wnt-calcium pathway (1). Among these, the canonical signal is the most studied in bone for its critical role in skeletal development and homeostasis in physiological and pathological conditions. Among all Wnt proteins, Wnt10b has the most crucial effect in bone formation in adult skeleton (2). Interestingly Wnt1 mutations have been linked to skeletal disorders in autosomal dominant early-onset osteoporosis and a recessive form of

Key words: Wnt1, bone, muscle, physical activity, mechanical loading

Mailing address: Maria Grano, PhD,
Full Professor, Department of Basic Medical Sciences,
Neuroscience and Sense Organs,
Section of Human Anatomy and Histology,
University of Bari, Piazza Giulio Cesare 11,
70124 Bari, Italy
e-mail: maria.grano@uniba.it

0393-974X (2015)

osteogenesis imperfecta (OI) (3-6).

OI is a genetic disorder characterized by severe bone fragility coupled in most cases with low bone mass and other symptoms including dentinogenesis imperfecta, short stature, blue sclerae and hearing loss in adulthood. The most common OI cases are dependent on defective synthesis of type I collagen, the most abundant protein of bone matrix. The genetic defect is caused by inherited mutations in Coll1a1 and Coll1a2, genes encoding the two α chains of collagen type I (7). Additionally, it has been reported that recessively inherited forms of OI may be caused by mutations of genes involved in type I collagen processing (8-14).

Very recently, a genetic study has proved that patients carrying Wnt1 homozygous truncation mutation are affected by a severe recessive form of OI, characterized by osteopenia, numerous fractures, short stature and deformities in long bones (5). The same work also studied patients with autosomal dominant osteoporosis, caused by heterozygous missense mutation in Wnt1, which displayed low bone mineral density but low propensity to vertebral and peripheral fractures, suggesting that the loss of Wnt1 synthesis could have a major impact on bone metabolism (5). Despite these human genetic evidences, there are few *in vitro* functional studies on the role of Wnt1 in osteoblast differentiation. Here we show that recombinant Wnt1 (r-Wnt1) strongly increases differentiation of bone marrow stromal cells into mature osteoblasts, as confirmed by enhanced number of cells positive for Alkaline phosphatase, the marker gene of osteoblast. We also prove that the bone anabolic effect of r-Wnt1 is mainly mediated through the up-regulation of Collagen I (Coll I) and Osteopontin (Opn) mRNA. In addition, our data show that endogenous Wnt1 synthesis in bone marrow is up-regulated by physical activity *in vivo*, and conditioned medium (CM) collected from myoblasts harvested from exercised mice up-regulates Wnt1 expression in osteoblast cell cultures. This work confirms that, among Wnt ligands, Wnt1 is another key molecule crucial in regulating bone metabolism and mediating the anabolic effect of physical activity on bone.

MATERIALS AND METHODS

Materials

Antibody anti-Wnt1 was from Abcam; Antibody anti-βActin was from Santa Cruz. Ascorbic acid, β-Glicerophosphate and Alkaline Phosphatase (ALP) staining kit were from Sigma Aldrich. Primers for qPCR are: ALP/S-aaacccagacacaagcattcc; ALP/AS-tccaccagcaagaagaagcc; Coll I/S-ggctcctgctcctcttag; Coll I/AS-acagtccagttcttcattgc; OPN/S cgccgaccaaggaaaactca; OPN/AS tgcttctgagatgggtcagg; GAPDH/S acaccagtagactccacgaca; GAPDH/AS acggcaaattcaacggcacag.

Exercise protocol

Two month-old C57BL/6 male mice were subjected to 5 weeks of normal activity (Control) or free wheel running activity (Exercise). The control mice were isolated each mouse in one cage, in order to avoid their tendency to fight with cage mates, preventing any exercise-mimetic activity. For exercise mice, endurance exercise was measured using in-cage voluntary running wheels, with electronic monitoring (VitalView) where time spent for running was measured. All mice were housed individually. Mice were weighed once a week during the experimental procedure. Animals were euthanized by cervical dislocation and their tissues were surgically excised.

Contact radiography

The right tibia was harvested and X-rayed by using contact radiography (Vista Scan Combi Durr Dental AG, Bietigheim-Bissingen). Scans were obtained by using a setting of 60 kV, 8 mA and 2.5 sec exposure time. The developed X-rays were scanned into a personal computer for image analysis. No direct measures were performed on the images.

Primary cell cultures

Primary Myoblasts were obtained from digestion of vastus lateralis specimens with a solution of Trypsin, Collagenase and $CaCl_2$, as described previously (15). Cells were then cultured until multinucleated, spontaneously contracting myotubes were formed. After 3 days, the conditioned media (CM) were collected. Bone marrow stromal cells, obtained by flushing bone marrow of 2-month old C57BL/6 mice, were cultured to induce osteoblast differentiation with α-MEM/10% FCS supplemented with 50 mg/ml ascorbic acid and 10^{-2} M β-glycerophosphate with or without CM from primary myoblast. Osteoblast were then subjected to mRNA and protein analysis.

RT–PCR

qPCR was carried out after RNA extraction using spin columns (RNasy, Qiagen) according to the manufacturer's instruction. By using SuperScript First-Strand Synthesis System kit (Invitrogen), the resulting cDNA (20 ng) was

subjected to quantitative PCR and, thereafter, to ITAQ SYBR Green Supermix with ROX kit (Bio-Rad) on an iCycler iQ5 Cromo4 (BioRad). Each transcript was assayed 3 times, and cDNA was normalized to murine Gapdh and quantitative measures were obtained using the $\Delta\Delta C_T$ method. Analyses were performed using unpaired Student's *t*-tests (Excel) for significant differences at $p<0.05$.

Immunofluorescence

Five nm thick bone sections were subjected to immunofluorescence staining, being incubated with primary antibody against Wnt1 (Abcam). Fluorescent-labeled goat anti-rabbit secondary antibody (Alexa-555 and Alexa-488, respectively; Invitrogen) were used for detection.

Western blot

Protein amounts from all samples were assessed using the BCA-kit (Biorad) followed by protein concentration normalization before all western blot experiments. 30 mg of cell proteins were subjected to SDS–PAGE. Subsequently proteins were transferred to nitrocellulose membranes (Hybond, Amersham). The blots were probed using primary antibodies, described in Materials section, and IRDye-labeled secondary antibodies (680/800CW) (LI-COR Biosciences). For immuno-detection, the Odyssey infrared imaging system was utilized (LI-COR Corp., Lincoln, NE). All data were normalized to background and loading controls.

RESULTS

Effects of r-Wnt1 on osteoblast differentiation marker genes

We tested the effect of r-Wnt1 on osteoblast differentiation *in vitro* using undifferentiated bone marrow stromal cells, cultured in the presence of ascorbic acid and β-glycerophosphate, treated with or without r-Wnt1 during a 3-weeks period of differentiation in culture. As shown in Fig. 1A, after 1 week of differentiation r-Wnt1 led to a 7.5-fold increase in the number of ALP positive colonies ($p<0.001$) at the dose of 10 ng/ml compared to control medium. In agreement with this, gene expression studies demonstrated that the treatment with r-Wnt1 (10 ng/ml) induced up-regulation of Alp mRNA at all 3-points of culture time course (Fig. 1B), with the greatest fold change increase after 1 week of osteoblast differentiation (+2.6 fold increase; $p<0.001$). Interestingly the expression of type Coll I, the most abundant protein in bone matrix, was strongly up-regulated by r-Wnt1 treatment at 1-week of osteoblast differentiation (+3.4 fold increase; $p<0.001$), as well as at 2-weeks of differentiation when Coll I expression still remained three times higher than untreated cells ($p<0.01$). r-Wnt1 treatment also increase the expression of Opn, a late stage marker of osteoblast differentiation. As expected Opn up-regulation was markedly evident at 3 weeks of differentiation when r-Wnt1 led to a 3-fold enhancement of its synthesis ($p<0.001$).

Physical exercise increases Wnt1 expression in bone marrow

Young male mice subjected to 5 weeks of free wheel running activity (Exercise) were monitored to ensure their physical performance. As showed in Fig. 2A, mice ran on average of 2,200 meters per day, while maintaining a maximum peak of 3.300 meters/day during the second week and a minimum peak of 1.700 meters/day during the fourth week. Radiographic analysis of tibia showed a generalized increase in radio-density of tibia of exercise mice compared with the control group (Fig. 2B), thus confirming the well know effect of physical activity on bone mineral density of long bone (16-17). Noteworthy, exercised mice displayed a notable increase in marrow *Wnt1* mRNA expression that was 2.5 times higher than control mice ($p<0.01$). In addition, we also showed that exercised mice displayed higher percentage of Wnt1 immune-stained bone marrow cells than those of control mice, demonstrating that its synthesis is upregulated by physical activity (Fig. 2D).

Conditioned medium from myoblasts of exercised mice increases Wnt1 expression in osteoblasts

We confirmed the effect of our exercise regimen on muscle tissue. As expected, exercised mice displayed 45% increase ($p<0.01$) in vastus lateralis weight/body weight compared with mice kept under resting conditions (Fig. 3A).

We cultured muscle cells after vastus lateralis digestion to recapitulate *in vitro* release of muscle-derived molecules following physical exercise. The conditioned medium (CM) collected from these myoblasts was used to evaluate its ability in regulating expression of Wnt1 during osteoblast differentiation.

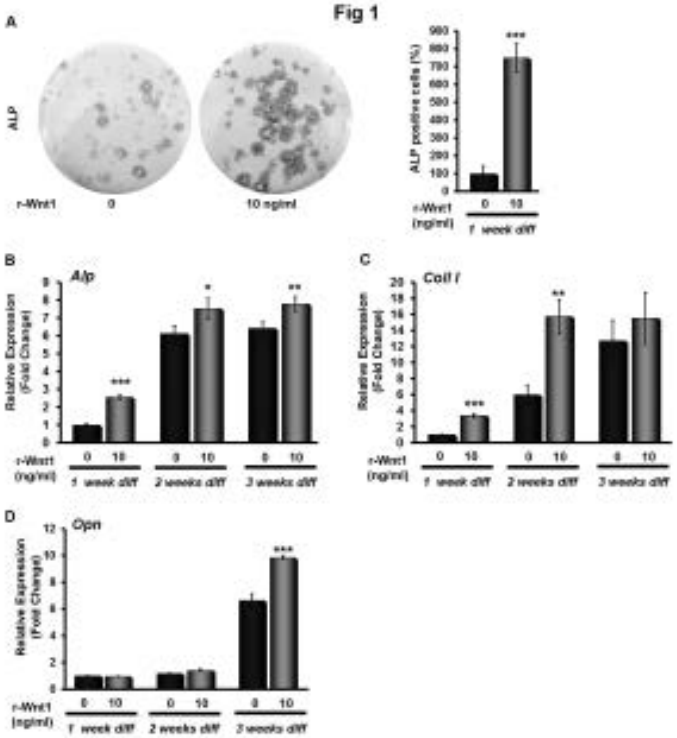


Fig. 1. *Effects of r-Wnt1 on osteoblast differentiation marker* genes. *Bone marrow stromal cells were grown in osteoblast differentiation medium in the presence of recombinant Wnt1 (r-Wnt1, 10 ng/ml). The percentage of alkaline phosphatase-positive colonies (10 days) (**A**) were calculated vs untreated cultures. Representative wells are shown. Osteoblast marker genes Alp (**B**), Coll I (**C**) and Opn (**D**) were evaluated (qPCR) following culture with r-Wnt1 (for the times indicated). ***: $p \leq 0.001$; **: $p \leq 0.01$; *: $p < 0.05$. Statistics: Unpaired Student's t-test. Data are presented as mean ± SEM.*

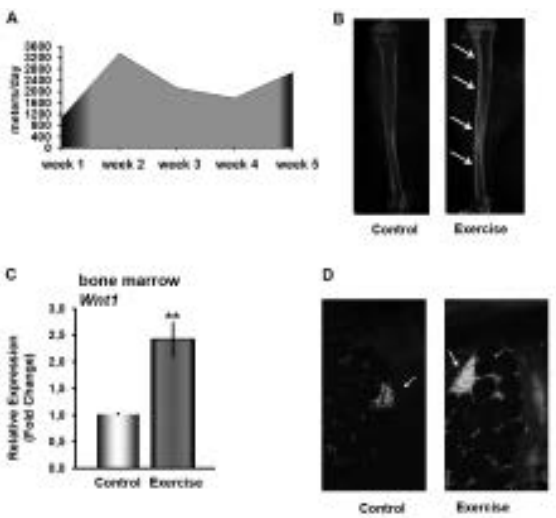


Fig. 2. *Physical exercise increases Wnt1 expression in bone marrow. **A**): Mice subjected to 5 weeks of free wheel running activity (Exercise) were monitored to ensure their physical performance and time spent for running was measured as showed in the representative graph; Contact radiographs of selected tibia from Control and Exercise mice. **B**): Arrows indicate increased radiodensity; **C**): Wnt1 mRNA expression (qPCR) in whole bone marrow isolated from tibiae of Control or Exercise mice. **D**): Fluorescent micrographs of tibial sections of Control or Exercise mice immunostained for Wnt1 [(green) nuclei labeled blue]. Magnification: 20x. **:p<0.01. Statistics: Unpaired Student's t-test; n=8-9 mice per group; data are presented as mean±SEM.*

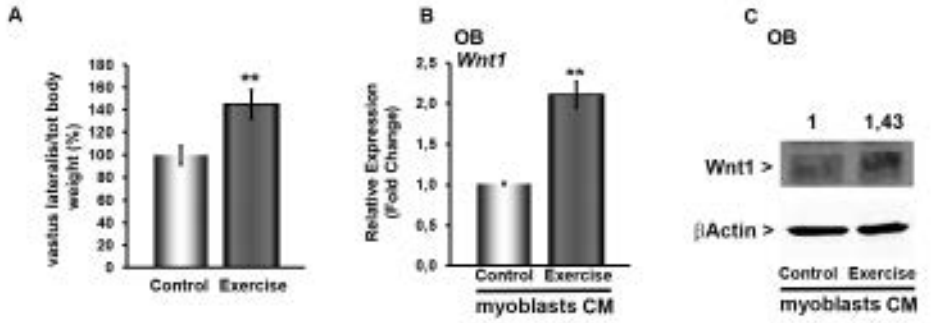


Fig. 3. ***Conditioned medium from myoblasts of exercised mice increases Wnt1 expression in osteoblasts. A****): Mice subjected to 5 weeks of free wheel running activity (Exercise) display increase in vastus lateralis weight/body weight; Wnt1 mRNA expression (qPCR) (**B**) and Wnt1 protein expression (Western blot) (**C**) in osteoblast cultures treated for 48 h with conditioned medium (CM) of myoblasts harvested from Control or Exercise mice. ******: p≤0.01; Statistics: Unpaired Student's t-test; data are presented as mean±SEM.*

Our result showed that the CM from exercised myoblasts increases 2.5-fold *Wnt1* mRNA expression (Fig. 3B). This result was also confirmed by analysing Wnt1 protein expression, which resulted 1.5-fold higher in osteoblast treated with myoblasts from exercised mice than those from control mice (Fig. 3C).

DISCUSSION

The work presented here supports the idea that Wnt1 plays a central role in regulating osteoblast differentiation and suggests that Wnt1 synthesis in bone may be amplified by physical exercise.

The link between Wnt1 and skeleton is only recently emerged through human genetic studies which identified that the human skeletal diseases of osteogenesis imperfecta is associated with mutations in Wnt1 (3-6). Thus far very few *in vitro* studies have suggested a Wnt1 direct action on osteoblasts. Among these, the study of Keupp et al (4), who created a Wnt1 protein model by mimicking Wnt1 mutation in OI, revealed that this altered Wnt1 protein failed to activate canonical LRP5-mediated WNT-regulated β-catenin signaling (4).

Furthermore, other authors proved that Wnt1 overexpression in human mesenchymal stem cells induces osteogenic differentiation in neighboring non-Wnt1-expressing wild type cells (18). Moreover, Wnt1 expression was found in brain, femur, spleen and bone marrow, with the highest expression in cells of B-cell lineage. Interestingly, by using lineage tracing experiments, authors found Wnt1 expression in a subset of osteocytes (5). Although animal models are generally required to improve the understanding of a molecule function, *in vivo* studies on Wnt1 knock out mice are not available, since these mice die in the neonatal period due to no midbrain and no cerebellum development (19). However, the only model currently existing to study the bone phenotype in the presence of Wnt1 dysfunction is represented by the Swaying mouse ($Wnt1^{sw/sw}$), which carries a single nucleotide deletion in the third exon of *Wnt1* (20-21). $Wnt1^{sw/sw}$ mice displayed spontaneous fractures and its osteopenic bone phenotype is caused by decreased osteoblast activity (21).

Data presented here give a contribution in the understanding of Wnt1 effects on osteoblast differentiation. In particular, we demonstrated that r-Wnt1 markedly increases differentiation of bone marrow stromal cells into mature osteoblasts, as confirmed by enhancement of the expression of Alkaline phosphatase, one of the marker gene of osteoblasts. Furthermore, the osteogenic ability of Wnt1 is also dependent on the up-regulation of mRNA levels of Collagen I, the most abundant bone matrix protein. Our data support the idea that Collagen I is one of the main target gene activated by Wnt1. Therefore, admittedly speculative, it is possible that a close association between collagen

I synthesis and Wnt1 expression could exist, given that the dysfunction of both molecules has been proven to be the cause of Collagenous Types and Non-Collagenous Types of OI, respectively (3-7). In agreement with our findings, researchers who investigated the functional role of the canonical Wnt pathway in fibrosis, found that Wnt-1 stimulated the transcriptional activity of Collagen1a2 in human dermal fibroblasts. Of note, in supernatants of fibroblasts stimulated with Wnt-1, a dose-dependent increase in collagen production was also found (22).

In our study we also show that Osteopontin mRNA levels are increased by r-Wnt when bone marrow precursors were differentiated into mature osteoblasts, supporting the involvement of Wnt1 in the bone anabolic response. Consistently, several studies showed increased Osteopontin expression in response to mechanical stimulation of bone cells in vitro (23-24) and, therefore, it has been defined a mechanically responsive gene (25).

Other experimental evidences demonstrated that Osteopontin might be responsible for bone loss in absence of loading, since Osteopontin null mice were resistant to unloading-induced bone loss (26). In line with the importance of loading and physical activity for bone, our *in vivo* and *in vitro* data on Wnt1 expression could be extremely relevant. Intriguingly, we found higher level of Wnt1 in bone marrow of mice subjected to physical activity, whose increase could be mediated *via* a not yet identified muscle-secreted factor, since osteoblast treated with myoblast-CM from exercised mice showed an increased synthesis of Wnt1, at both mRNA and protein levels. Very recently, several lines of evidence established that skeletal muscle is an endocrine organ producing and releasing myokines, which might be effectors of the positive outcome on the body triggered by physical activity (27). Our findings support the novel concept that muscle and bone not only communicate via osteocyte-dependent mechanical loading, but also that muscle can secrete factor(s) that can act in a paracrine fashion to support bone accrual and maintenance. The identity of the muscle-released factor which is able to increase Wnt1 synthesis after physical activity still remains unknown but future investigations on this topic could reveal new insights in the bone-muscle connection.

In conclusion, our findings highlight Wnt1 as one of the key molecule involved in the regulation of bone formation and as one of mediators of beneficial effect on bone triggered by physical activity.

REFERENCES

1. Day TF, Guo X, Garrett-Beal L, Yang Y. Wnt/beta-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. Dev Cell 2005 May; 8(5):739-50.
2. Bennett CN, Longo KA, Wright WS, Suva LJ, Lane TF, Hankenson KD, MacDougald OA. Regulation of osteoblastogenesis and bone mass by Wnt10b. Proc Natl Acad Sci USA 2005; 102(9):3324-9.
3. Fahiminiya S, Majewski J, Mort J, Moffatt P, Glorieux FH, Rauch F. Mutations in WNT1 are a cause of osteogenesis imperfecta. J Med Genet 2013; 50:345-8.
4. Keupp K, Beleggia F, Kayserili H, et al. Mutations in WNT1 cause different forms of bone fragility. Am J Hum Genet 2013; 92:565-74.
5. Laine CM, Joeng KS, Campeau PM, et al. WNT1 mutations in early-onset osteoporosis and osteogenesis imperfecta. N Engl J Med 2013; 368:1809-16.
6. Pyott SM, Tran TT, Leistritz DF, et al. WNT1 mutations in families affected by moderately severe and progressive recessive osteogenesis imperfecta. Am J Hum Genet 2013; 92:590-7.
7. Marini JC, Forlino A, Cabral WA, et al. Consortium for osteogenesis imperfecta mutations in the helical domain of type I collagen: regions rich in lethal mutations align with collagen binding sites for integrins and proteoglycans. Hum Mutat 2007; 28(3):209-21.
8. Barnes AM, Chang W, Morello R, et al. Deficiency of cartilage-associated protein in recessive lethal osteogenesis imperfecta. N Engl J Med 2006; 355:2757-64.
9. Morello R, Bertin TK, Chen Y, et al. CRTAP is required for prolyl 3- hydroxylation and mutations cause recessive osteogenesis imperfecta. Cell 2006; 127:291-304.
10. Cabral WA, Chang W, Barnes AM, et al. Prolyl 3-hydroxylase 1 deficiency causes a recessive

metabolic bone disorder resembling lethal/severe osteogenesis imperfecta. Nat Genet 2007; 39:359-65.
11. Baldridge D, Schwarze U, Morello R, et al. CRTAP and LEPRE1 mutations in recessive osteogenesis imperfecta. Hum Mutat 2008; 29:1435-42.
12. Barnes AM, Carter EM, Cabral WA, et al. Lack of cyclophilin B in osteogenesis imperfecta with normal collagen folding. N Engl J Med 2010; 362:521-8.
13. Christiansen HE, Schwarze U, Pyott SM, et al. Homozygosity for a missense mutation in SERPINH1, which encodes the collagen chaperone protein HSP47, results in severe recessive osteogenesis imperfecta. Am J Hum Genet 2010; 86:389-98.
14. Kelley BP, Malfait F, Bonafe L, et al. Mutations in FKBP10 cause recessive osteogenesis imperfecta and Bruck syndrome. J. Bone Miner. Res. 2011; 26:666–72.
15. Colaianni G, Cuscito C, Mongelli T, et al. Irisin enhances osteoblast differentiation in vitro. Int J Endocrinol 2014; 2014:902186.
16. Baxter-Jones AD, Kontulainen SA, Faulkner RA, Bailey DA. A longitudinal study of the relationship of physical activity to bone mineral accrual from adolescence to young adulthood. Bone 2008; 43(6):1101-7.
17. Andreoli A, Celi M, Volpe SL, Sorge R, Tarantino U. Long-term effect of exercise on bone mineral density and body composition in post-menopausal ex-elite athletes: a retrospective study. Eur J Clin Nutr 2012; 66(1):69-74.
18. Liu G, Vijayakumar S, Grumolato L, Arroyave R, Qiao H, Akiri G, Aaronson SA. Canonical Wnts function as potent regulators of osteogenesis by human mesenchymal stem cells. J Cell Biol 2009; 185(1):67-75.
19. McMahon AP, Bradley A. The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. Cell 1990; 62:1073-85.
20. Thomas KR, Musci TS, Neumann PE, Capecchi MR. Swaying is a mutant allele of the proto-oncogene Wnt-1. Cell 1991; 67:969-76.
21. Joeng KS, Lee YC, Jiang MM, et al. The swaying mouse as a model of osteogenesis imperfecta caused by WNT1 mutations. Hum Mol Genet 2014; 23(15):4035-42.
22. Akhmetshina A, Palumbo K, Dees C, Bergmann C, Venalis P, Zerr P, et al. Activation of canonical Wnt signalling is required for TGF-β-mediated fibrosis. Nat Commun 2012; 3:735.
23. Harter LV, Hruska KA, Duncan RL. Human osteoblast-like cells respond to mechanical strain with increased bone matrix protein production independent of hormonal regulation. Endocrinology 1995; 136(2):528-35.
24. Kubota T, Yamauchi M, Onozaki J, Sato S, Suzuki Y, Sodek J. Influence of an intermittent compressive force on matrix protein expression by ROS 17/2.8 cells, with selective stimulation of osteopontin. Arch Oral Biol 1993; 38(1):23-30.
25. Toma CD, Ashkar S, Gray ML, Schaffer JL, Gerstenfeld LC. Signal transduction of mechanical stimuli is dependent on microfilament integrity: identification of osteopontin as a mechanically induced gene in osteoblasts. J Bone Miner Res 1997; 12(10):1626-36.
26. Ishijima M, Tsuji K, Rittling SR, Yamashita T, Kurosawa H, et al. Osteopontin is required for mechanical stress-dependent signals to bone marrow cells. J Endocrinol 2007; 193(2):235-43.
27. Colaianni G, Cuscito C, Mongelli T, et al. The Myokine Irisin Increases Cortical Bone Mass. Proc Natl Acad Sci USA 2015; 112(39):12157-62.

EFFECT OF PLATELET RICH PLASMA CONCENTRATION ON SKELETAL MUSCLE REGENERATION: AN EXPERIMENTAL STUDY

M. CIANFORLINI[1], M. MATTIOLI-BELMONTE[1], S. MANZOTTI[1], E. CHIURAZZI[2], M. PIANI[2], F. ORLANDO[3], M. PROVINCIALI[3] and A. GIGANTE[1]

[1]Department of Clinical and Molecular Sciences, School of Medicine, Università Politecnica delle Marche, Ancona, Italy; [2]Department of Trasfusion Medicine, Ospedali Riuniti, Ancona, Italy; [3]Experimental Animal Models for Aging Research, Scientic Technological Area, IRCCS-INRCA, Ancona, Italy

The first two authors contributed equally to this work.

Skeletal muscle injuries are common causes of severe long-term pain and physical disability, accounting for up to 55% of all sports injuries. The phases of the healing processes after direct or indirect muscle injury are complex but clearly defined and include well-coordinated steps: degeneration, inflammation, regeneration, and fibrosis. Despite this frequent occurrence and the presence of a body of data on the pathophysiology of muscle injuries, none of the current treatment strategies have shown to be really effective in strictly controlled trials. Platelet-rich plasma (PRP) is a promising alternative approach based on the ability of autologous growth factors (GFs) to accelerate tissue healing, improve muscular regeneration, increase neovascularization and reduce fibrosis. The present study is focused on the use of different concentrations of PRP as a source of GFs. Unilateral muscle lesions were created on the longissimus dorsi muscle of Wistar rats. Twenty-four h after surgical trauma, the lesion was filled with an intramuscular injection of PRP at 2 different concentrations. A group of rats were left untreated (controls). Animals were sacrificed at 3, 15 and 60 days from surgery. Histological, immunohistochemical and histomorphometric analyses were performed to evaluate muscle regeneration, neovascularization, fibrosis and inflammation. The PRP-treated muscles showed better muscle regeneration, more neovascularization and a slight reduction of fibrosis compared with the control muscles in a dose dependent manner. However, further studies also assessing pain and functional recovery are scheduled.

Skeletal muscle injuries, mainly contusions and strains, are common causes of severe long-term pain and physical disability. They account for up to 55% of all sports injuries and for 31% of all damages in élite football (soccer) players. Ninety-two percent of the lesions occurring in football (soccer) players affects the four major muscle groups of the lower limbs: hamstrings 37%, adductors 23%, quadriceps 19% and calf muscles 13%. As many as 96% of all muscle injuries in football (soccer) occur in non-contact situations and 16% of these are re-injuries and they are associated with a longer absence from competition (1, 2).

Muscle injury is a challenging problem in

Key words: muscle Injuries, platelet rich plasma, growth factors, animal study

Mailing address: Dr. Antonio Gigante,
Department of Clinical and Molecular Sciences,
Università Politecnica delle Marche,
Via Tronto 10/ 60126, Ancona, Italy
Tel.: +39 071 220 6069
Fax: +39 071 220 6069
e-mail: a.gigante@univpm.it

0393-974X (2015)

traumatology, as injured muscles heal very slowly and often with incomplete functional recovery hence the critical importance of a correct evaluation, diagnosis and therapy of these disorders. Indeed, the variety of criteria that available makes it difficult to develop a single classification of muscle injuries. Their severity is defined by the amount of muscle tissue involved and by the extent and the location of the effusion. Different classification systems are published in literature (3, 4, 5) but there is little consistency between studies and daily practice (6, 7).

In sport, muscle lesion treatment is aimed to re-enable the athlete to train and compete as soon as possible and without complications. Muscle injuries of lesser severity usually benefit from nonsurgical treatment such as PRICE (Protection, Rest, Ice, Compression and Elevation), non-steroidal anti-inflammatory drugs (NSAIDs), early mobilization, physical therapy (active and passive modalities) and functional rehabilitation protocols. As an alternative, the surgical re-approximation of muscle tears is a possibility. All these therapeutic approaches seem inadequate in extensive muscle lesions and/or in sportsmen with higher functional demand (8).

Phases of the healing processes after direct or indirect muscle injury are well defined: the initial degenerative/necrotic phase is mostly characterized by the formation of hematoma; the subsequent inflammatory/cell response and the repair/fibrosis phases are strictly interrelated and time-dependent. Local swelling and hematoma formation occur rapidly after injury, with clot formation and associated platelet degranulation, which leads to the local release of growth factors (GFs) and cytokines that in turn determine the neutrophils and macrophages chemotactic migration in the lesion site. Subsequently, myogenic precursor cells (i.e. satellite cells) undergo activation and proliferation, also facilitated by GFs release (9). Once activated, satellite cells that are located between the basal lamina and the plasma membrane of each myofiber proliferate and differentiate into multinucleated myotubes and even myofibers. Many of these cells are able to fuse with existing necrotic myofibers and may prevent the complete muscle fiber degeneration. In most cases, the healing process results in the formation of a muscle characterized by an area of fibrotic scar tissue (differing in size depending on the extension of the primary lesion) with an inadequate replenish of its functional capacity. In fact, the regenerative capacity of a muscle lesion varies widely and depends on the severity of the trauma (10). The chance of a complete *restitutio ad integrum* is proportional to the lesion extent and dependent on pathophysiological processes that characterize the early post-injury phase (i.e. 0-72 hours after lesion).

The role of GFs in the process of satellite cell activation and muscle regeneration is of outmost importance. Barriers to complete muscle recovery following injury are scarring and fibrosis, thus regulation of fibrosis is one of the goals of PRP therapy in the management of muscle lesions (8).

Platelet-rich plasma (PRP) is an autologous concentration of human platelets to supra-physiologic levels that function as a natural reservoir for GFs, including platelet-derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factor-beta 1 (TGF-β1), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF) and insulin-like growth factor1 (IGF-1) (11). Most of these growth factors have shown the capacity to stimulate myogenesis both *in vitro* and *in vivo* (12).

Even though PRP is commonly used in orthopaedic practice to enhance healing in sports-related skeletal muscle, tendon, and ligament injuries (10, 13), its use is mainly based on limited experimental data; no meta-analysis studies or randomized controlled trials have been conducted to afford their safe and effective use in muscle lesions (13, 14). The few *in vivo* studies have shown that GFs are able to improve muscle regeneration and increase the muscle strength after a trauma.

Experimental studies on animal models showed that IGF-1, bFGF and nerve growth factor (NGF) are potent stimulators of myoblast proliferation and fusion (15).

Due to the short half-life and rapid clearance of GFs, injured muscles need to be treated with high concentrations of these molecules. The present study is therefore focused on the use of different concentrations of PRP in an experimental animal model.

The principal aim of the present experimental *in vivo* study is a morphological and morphometrical evaluation of the PRP effects on full thickness

muscle lesions in an animal model to verify a relationship between concentrations of GFs and muscle regeneration and fibrosis.

MATERIALS AND METHODS

Thirty-six male Wistar rats (340±60 g/BW; PLT=4.5±0.3*10^5/mmc) were used (Experimental Animal Models for Aging Units Research Department, I.N.R.C.A./ I.R.R.C.S., Ancona, Italy). The rats were inbred, therefore they are considered genetically identical. The policies and procedures of their use and maintenance were in accordance with those detailed by the directive n. 86/609/ CEE regarding animal care and experimental usage.

PRP Preparation

Briefly, blood necessary for PRP preparation was obtained by intracardiac puncture from 9 rats using a 10 mL syringe containing citrate phosphonate dextrose (CPD) in ratio 5:1. Two different concentrations of platelets were achieved. In the first group (A) blood was centrifuged at 3000 rpm for 6 min (ThermoFisher SCIENTIFIC SL16/16R 2010, radius=14 cm), plasma was removed and the platelet-rich fraction was used for a second centrifugation at 5000 rpm for 10 min (Heraeus SEPATECH GmbH LABOFUGE A, radius=12 cm). The pellet from the second centrifugation was diluted with plasma until the platelet concentration became 1,200x10^6 platelets/mmc. In the second group (B) blood was centrifuged at 2400 rpm for 6 min (ThermoFisher SCIENTIFIC SL16/16R 2010, radius=14 cm), plasma was removed and the platelet-rich fraction was used for a second centrifugation at 5000 rpm for 10 min (Heraeus SEPATECH GmbH LABOFUGE A, radius=12 cm). The pellet from the second centrifugation was saved and diluted with supernatant until the platelet concentration became 1.700×10^6 platelets/mmc.

Collected PRP was stored at -80°C until use. The syngeneic nature of the inbred rats allowed us to consider the blood of one animal autologous to blood from another animal.

Surgical Procedure and Sample Preparation

Animals were anesthetized with ketamine (40 mg/ Kg) and xylazine (5 mg/kg) intramuscular puncture and placed prone on a warm pad (38.5ºC). A sterile airway was maintained throughout the procedure. Unilateral cutaneous incisions (3 cm in length) were performed in the paravertebral region, then, using a standard pincer technique, muscular tear lesions (0.7x0.3 cm) were executed on the longissimus dorsi muscle (Fig. 1) as performed in a previous experimental study (16). This location was chosen since the presence of the supraspinatus ligament and fascia allow to carry out the wounds and prevents rats from interfering (i.e. biting or scratching) with the surgical treatment. In all cases muscle was left unsutured, the fascia and skin were closed with a standard approach with a non-absorbable suture wire in order to identify the repair site at the time of necropsy. Animals were maintained with a normal diet and no forced exercise was induced.

PRP intramuscular injection in the lesion sites were performed 24 h after the surgical trauma: 9 rats received the lowest PRP concentration (group A), 9 rats received the highest PRP concentration (group B) and 9 rats were

Table I. *Histological qualitative score.*

SCORE	**Vascularization**	**Muscle regeneration**	**Inflammation**	**Fibrosis**
0	no evidence of neovascularization	no new muscular tissue	more than 50% of the field occupied by inflammatory cells	more than 50% of the section displaying fibrotic aspect
1	less than 25% of the microscopic field covered with new vessels	less than 25% of the microscopic section filled with new muscular tissue	25%-50% of the field occupied by inflammatory cells	25%-50% of section displaying fibrotic features
2	lesion with 25%-50% of the field covered with new vessels	lesion with 25%-50% of the section filled with new muscular tissue	less than 25% of the microscopic field occupied by inflammatory cells	less than 25% of the microscopic section displaying fibrotic aspect
3	repair site with more than 50% of the field covered with new vessels.	repair site with more than 50% of the section filled with new muscular tissue	no evidence of inflammation	no evidence of fibrosis

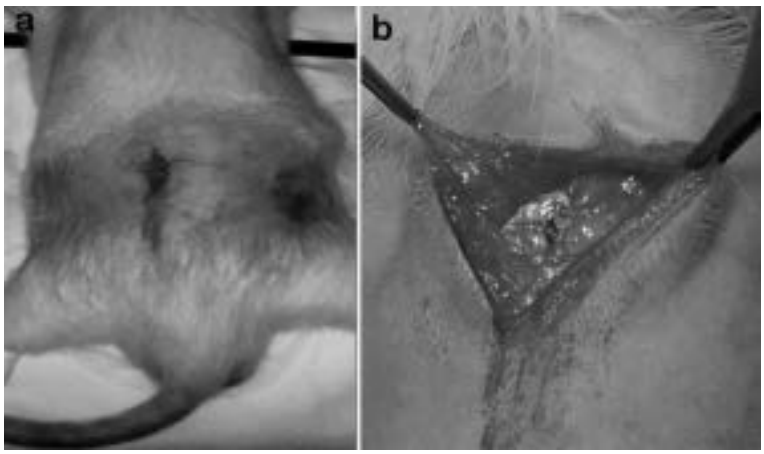


Fig. 1. *Male Wistar rat: unilateral cutaneous incision (3 cm in length) was performed in the paravertebral region (**a**); a muscular tear lesion (0.7 x 0.3 cm) was executed on the longissimus dorsi muscle using a standard pincer technique (**b**).*

left untreated and used as controls (CTRL).

Three animals per group were sacrificed each time at an interval of 3, 15 and 60 days from surgery. The experimental sites were dissected and collected samples were fixed in 4% formaldehyde embedded in paraffin, sectioned (5 µm) and stained with haematoxylin-eosin, Van Gieson's trichrome stains and Sirius-Red for histological assessment.

Histological evaluation

Sections were evaluated by 2 different observers in a blinded manner. Each examiner considered three fields for every section (5 slices for lesion). Both longitudinal and transverse sections were observed at 10x magnification evaluating muscle regeneration, neovascularization, inflammation and fibrosis by means of a qualitative score summarised in Table I (16-18).

Histomorphometric evaluation

Computerized morphometric analysis was performed with use of the Leica Q5OOMC Image Analysis System Software (Leica Leitz DMRBE, Cambridge, England) as previously described (7). Analysed field was briefly selected and acquired on the screen. The camera images were then digitized and modified in a binary way to make them suitable for measurement.

Similar to histological analysis the measured parameters were muscle regeneration, neovascularization, inflammation and fibrosis. All morphometric steps, except for the selection of the first microscopic field were computerized. After standard filtration procedures for background smoothing, the system identified the regenerating muscle fibres and the other parameters based on a threshold value set by the operator showing them in colour on the screen. Editing facilities for the correction of automatic identification were used. Zoom-in allowed a closer inspection of the samples.

On the basis of a calibration factor determined by a suitable procedure in the setup menu, the system calculates the fraction area (Aa%) filled by the selected parameters (muscle regeneration, neovascularization, inflammatory cells and fibrosis) on the whole field. Five fields were studied for each section. Data are expressed as mean±Standard Deviation (SD). The different parameters were evaluated each time selecting the area of interest.

Immunohistochemical analysis

Polysine® slides (Menzel-Gläser, Braunschweig, Germany) were used for immunohistochemical analysis. Dewaxing, rehydration and antigen unmasking were performed with EnVision™ FLEX Target retrieval Solution High pH (Dako, Carpinteria, CA, USA) by PT Module (Lab Vision Corporation, CA, USA) according to manufacturer's instruction. Endogenous peroxidase activity was quenched by incubating the sections in 3% H2O2 for 10 min at Room Temperature (RT).

Sections were then incubated with the monoclonal antibodies anti-MyoD (5.8A) and anti-Myogenin (5FD) (both Santa Cruz Biotechnology, CA, USA) diluted 1.150 in Antibody Diluent with Background Reducing Components (Dako) for 1 h at RT in a humidified atmosphere. Reaction was displayed by

Table II. *Histological score analysis.*

	3 days			15 day			60days		
	Group A	Group B	CTRL	Group A	Group B	CTRL	Group A	Group B	CTRL
Neovascularization	2.5±0.6	2.7±0.3	2.4±0.5	2.1±0.6	2.3±0.3	2.0±0.5	1.8±0.4	1.7±0.6	1.8±0.7
Muscle regeneration	2.0±0.5	2.2±0.4	1.5±0.5	2.7±0.3	2.8±0.2	2.3±0.4	1.5±0.2	1.6±0.7	1.4±0.3
Fibrosis	1.5±0.6	1.4±0.6	1.7±0.5	1.6±0.3	1.5±0.5	1.4±0.4	1.4±0.6	1.2±0.2	1.7±0.4
Inflammation	2.2±0.5	2.5±0.6	2.0±0.4	1.6±0.3	1.7±0.5	1.5±0.8	1.5±0.2	1.5±0.7	1.6±0.6
Score analysis is explained in Material and Methods									

LSAB®Plus System-HRP DAB+ kit (Dako) according to the manufacturer's instructions. Sections were counterstained, dehydrated, clarified and mounted with Mayer haematoxylin (Bio-Optica SpA, Milano, Italy). Negative control was represented by primary antibody untreated sections. Reaction was examined with a light microscope (Nikon Eclipse 600).

Statistical Analysis

Statistical analysis of histomorphometric data was performed with Wilcoxon non parametric test. The level of statistical significance was established at $p<0.05$.

RESULTS

Gross examination did not show differences between PRP treated or untreated muscle lesions. Histological score evaluation (Table II) evidenced that muscular regeneration was greater in PRP treated samples in comparison with controls at the earlier time points (i.e. 3 and 15 days), whilst it was comparable at 60 days after surgery.

Comparison of the two PRP treated groups by histomorphometric analysis (Fig. 2) showed that in the animals treated with PRP at a higher concentration of growth factors (group B), muscular regeneration was higher both at 3 at (A: 55±8% *vs* B:75±6%; $p<0.05$) and 15 days after surgery (A: 80±8% *vs* B: 90±6%; $p<0.05$), while no differences were detected 60 days after surgery (A: 52±8% *vs* B: 56±6%; $p<0.05$).

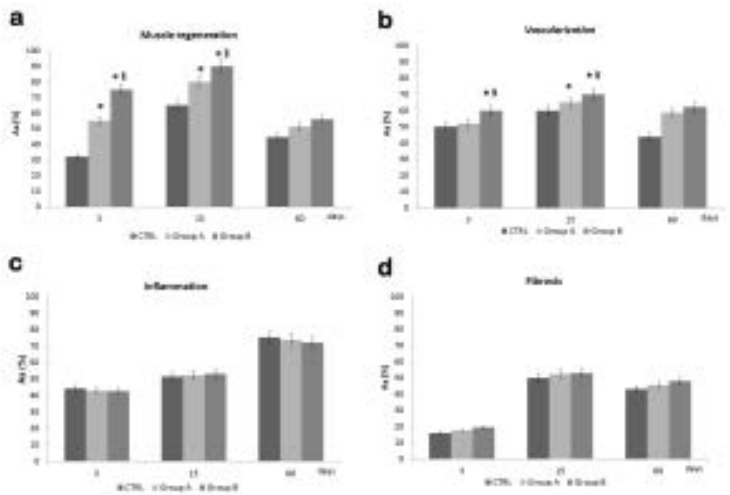


Fig. 2. *Histograms of the histomorphometric analysis performed at 3, 15 and 60 days after surgery and evaluating muscle regeneration (**a**); neovascularization (**b**); inflammation (**c**); fibrosis (**d**). Data are expressed as the fraction area (**Aa%**) occupied by the selected parameters; mean value±standard deviation are reported. ***:** $p<0.05$ vs CTRL; §: $p<0.05$ Group B vs Group A.*

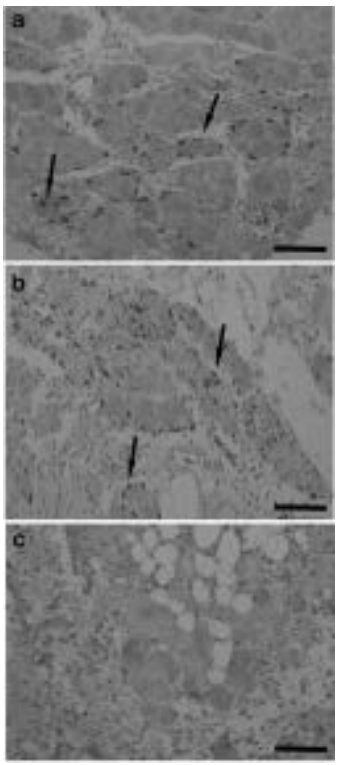


Fig. 3. *Immunoistochemical detection of MyoD at 3 days after surgery. MyoD- positive cells (→) located inside the basal lamina of myofibers were detected in Group A (**a**) and Group B (**b**) treated samples. No staining was present in untreated control samples (**c**). Scale bar 100µm.*

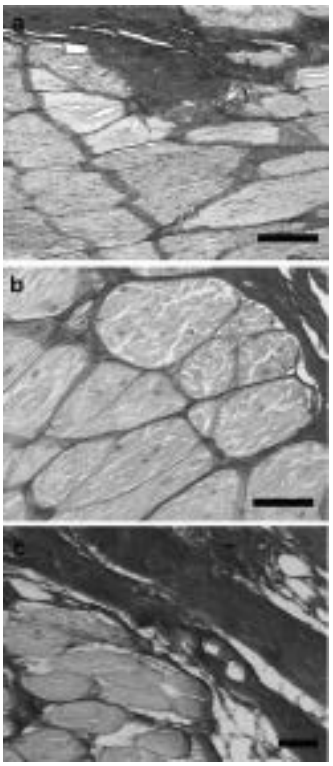


Fig. 4. *Histological sections of longissimus dorsi muscle of Wistar rats treated with lowest PRP concentration (**a**); highest PRP concentration (**b**); untreated (**c**); at 60 days after surgery. Sirius red stain. Scale bar 100µm.*

This observations was corroborated by immunohistochemical analyses performed at 3 days after surgery. In PRP treated lesions, the higher Myogenin positivity and MyoD immune-staining were directly proportional to the concentration of GFs (Fig. 3).

Data on muscle regeneration go hand in hand with those of neovascularization (Fig. 2b). This feature was greater in PRP treated samples in comparison with controls at the earlier time points of 3 and 15 days, but it was comparable 60 days after surgery. Also in this case the use of a higher concentration of GFs significantly affect neovascularization at 3 and 15 days after surgery, but it seems ineffective after 60 days.

As far as inflammation is concerned, no differences were detected between all the groups throughout the follow-up, even though a significant reduction of inflammatory cells was observed at 60 days from surgery (Fig. 2c)

At last a reduction in fibrosis was observed at 15 days after surgery, with no significant among the groups (Fig. 2d), even though faint differences in Sirius Red staining were detected at 60 days after surgery (Fig. 4).

DISCUSSION

Despite the frequency of skeletal muscle injuries and the availability of a body of data on their pathophysiology, none of the treatments adopted to date have been shown to be really effective in strictly controlled trials (8, 20).

One possible explanation for this apparently paradoxical situation is the broad heterogeneity of skeletal muscle injuries and in consequence

variability in severity. Furthermore, most existing muscle injury treatments are based on limited experimental data and/or were only empirically tested.

The aims of a proper muscle injury treatment are 1): limit the consequences of the damage on tissues involved in the trauma; 2): prevent future impairments; 3): ensure a rapid return to competitive activity to athletes while respecting compulsory biological healing time. These three points are strictly connected and conditioned to treatment(s) performed within the first 24-48 h from trauma. Growth factor activities occur at this time, creating an environment for muscle regeneration, neovascularization, inflammation and fibrosis.

Hammond et al. showed the capacity of PRP to promote and accelerate myogenesis in an experimental study investigating the biomechanical and biochemical effects in rat muscle injuries (21).

In a previous experimental study of muscle injury in a rat model, the effects of a platelet-rich fibrin matrix (PRFM) in the regeneration of damaged muscle tissue were histologically and immunohistochemically evaluated (16). This morphological study showed that the use of PRFM could improve muscle regeneration and long-term vascularization, suggesting that autologous PRFM may be a suitable and useful tool in the clinical treatment of muscle injuries (16).

A side effect of the use of PRP and/or related products (e.g. PRFM) may be the occurrence of fibrosis. Visser et al. demonstrated that PRFM *in vitro* contains significantly higher concentrations of TGF-β1 compared with whole blood concentrate of similar volume (22). TGF-β1 has the ability to significantly increase connective cell proliferation over time, thus generating fibrotic tissue. Indeed, in an *in vivo* study, no increase in fibrotic tissue formation was observed during PRFM treatment in comparison with controls, suggesting that the amount of TGF-β released *in vivo* by PRFM might not be sufficient for this occurrence (16).

In this respect, Huard et al. recently performed an additional experimental study combining PRP and losartan, a drug commonly used to treat hypertension. This mixture improved skeletal muscle healing by enhancing angiogenesis and follistatin expression and reducing the expression of phosphorylated Smad 2/3 and fibrosis development. The latter aspect was related to the reduction of TGF-β1 expression by losartan and suggested that its use improves the effect of PRP therapy on muscle healing after a contusion injury (23-25). More recently, Kelc et al. suggesting that PRP could not only prevent fibrosis but could also stimulate muscle commitment, especially when combined with a TGF-β inhibitor (26) reported a similar in vitro approach. Only a few randomized controlled clinical trials have been published, but none of them has ever compared different concentrations of growth factors (20).

In the present experiment on a Wistar rat model, we performed unilateral muscular lesion by a standard pincer technique through an open surgical approach in the paravertebral region. In agreement with previous experiences, this anatomical site was chosen since the supraspinatus ligament, the interspinatus ligament and fascia allow to circumscribe the wounds and moreover prevent rats from interfering (i.e. biting or scratching) with surgical treatment (16). We considered this surgical approach a suitable procedure to obtain a well-defined muscle tear rather than subjecting the muscle to a direct contusion or eccentric and tetanic contractions as performed in other experimental models (8, 16, 27).

With the proposed procedure it was possible to obtain a muscle injury without causing ischemic necrosis or thermal damages (16). Moreover, this approach allowed better localisation of the lesion in comparison to other animal model obtaining a reliable histological assessment (16).

In order to simulate a clinical approach closely, we injected two different concentration of PRP intramuscularly 24 h after the surgical trauma and we evaluated by means of histological and immunohistochemical analyses, the dose-dependent effects. Our histological results confirmed the ability of PRP in muscle healing and showed that the increase in PRP concentrations (i.e. GFs) in damaged muscle tissue fastens the process of tissue regeneration as well as neovascularization (16, 21, 23). Immunohistochemical data further strengthened this hypothesis detecting MyoD- and myogenin-positive cells, located both inside the basal lamina of the fiber and in the interstitial spaces in the muscle sacrificed at three days and in a dose-

dependent manner. It is well known that MyoD and myogenin play a key regulatory role in the processes of plasticity, adaptation and regeneration in adult muscle (24). At last, no significant side effects related to a higher dose of GFs were detected.

In conclusion, the present study confirmed previous data about the usefulness of PRP in the treatment of muscle injury and demonstrated that this process is dependent on the concentration of administered GFs. Even though these experimental results are lacking in data on pain and functional recovery, they open a clinical debate on the appropriate dose of PRP for sportsmen muscle lesion treatment.

REFERENCES

1. Feeley BT, Kennelly S, Barnes RP, et al. Epidemiology of National Football League training camp injuries from 1998 to 2007. Am J Sports Med 2008; 36:1597-603.
2. Lopez V Jr, Galano GJ, Black CM, et al. Profile of an American amateur rugby union sevens series. Am J Sports Med 2012; 40:179-184.
3. O'Donoghue DH. The Treatment of Injuries to Athletes. W.B. Saunders, Philadelphia 1962.
4. Stoller DW. MRI in orthopaedics and sports medicine. 3rd edn. Philadelphia: Wolters Kluwer/Lippincott 2007.
5. Askling CM, Tengvar M, Saartok T, et al. Acute first-time hamstring strains during high speed running: a longitudinal study including clinical and magnetic resonance imaging findings. Am J Sports Med 2007; 35:197-206.
6. Maffulli N, Oliva F, Frizziero A, et al. ISMuLT Guidelines for muscle injuries. Muscles Ligaments Tendons J. 2014; 3(4):241-9.
7. Mueller-Wohlfahrt HW, Haensel L, Mithoefer K, et al. Terminology and classification of muscle injuries in sport: the Munich consensus statement. Br J Sports Med 2013; 47:342-530.
8. Mosca MJ, Rodeo SA. Platelet-rich plasma for muscle injuries: game over or time out? Curr Rev Musculoskelet Med. 2015; 8(2):145-53.
9. Kasemkijwattana C, Menetrey J, Bosch P, et al. Use of growth factors to improve muscle healing after strain injury. Clin Orthop Relat Res 2000; 370:272-85.
10. Järvinen TA, Järvinen TL, Kääriäinen M, et al. Muscle injuries: optimising recovery. Best Pract Res Clin Rheumatol 2007; 21:317-31.
11. Castillo TN, Pouliot MA, Kim HJ, et al. Comparison of growth factor and platelet concentration from commercial platelet-rich plasma separation systems. Am J Sports Med 2011; 39:266-71.
12. Velloso CP. Regulation of muscle mass by growth hormone and IGF-I. British Journal of Pharmacology 2008; 154:557-68.
13. Menetrey J, Kasemkijwattana C, Day CS, et al. Growth factors improve muscle healing in vivo. J Bone Joint Surg Br 2000; 82:131-7.
14. Benazzo F, Perticarini L. Use of "tissue bioengineering": clinical applications on muscles. G.I.O.T. 2010; 36:206-10.
15. Anitua E, Andia I, Ardanza B, et al. Autologous platelets as a source of proteins for healing and tissue regeneration. Thromb Haemost 2004; 91(1):4-15.
16. Gigante A, Del Torto M, Manzotti S, et al. Platelet rich fibrin matrix effects on skeletal muscle lesions: an experimental study. J Biol Regul Homeost Agents 2012; 26:475-84.
17. Khattak MJ, Ahmad T, Rehman R, et al. Muscle healing and nerve regeneration in a muscle contusion model in the rat. J Bone Joint Surg Br 2010; 92(6):894-9.
18. Ranly DM, Lohmann CH, Andreacchio D, et al. Platelet-rich plasma inhibits demineralized bone matrix-induced bone formation in nude mice. J Bone Joint Surg 2007; 89:139-47.
19. Gigante A, Chillemi C, Potter K.A, et al. Elastic fibers of musculoskeletal tissues in bovine Marfan syndrome: a morphometric study. J Orthop Res1999; 17(4):624-8.
20. Shariff A, Razif MAM, Yusof A, et al. Platelet-rich plasma (PRP): an adjuvant to hasten hamstring muscle recovery. A randomized controlled trial protocol. BMC Musculoskeletal Disorders 2012; 13:138.
21. Hammond JW, Hinton RY, Curl LA, et al. Use of autologous platelet-rich plasma to treat muscle strain injuries. Am J Sports Med 2009; 37:1135-42.
22. Visser LC, Arnoczky SP, Caballero O, et al. Platelet-rich fibrin constructs elute higher concentrations of transforming growth factor-β1 and increase tendon cell proliferation over time when compared to

blood clots: a comparative in vitro analysis. Vet Surg 2010; 39:811-7.
23. Huard J, Li Y, Fu FH. Muscle injuries and repair: current trends in research. J Bone Joint Surg Am 2002; 84:822-32.
24. Terada S, Ota S, Kobayashi M, et al. Use of an antifibrotic agent improves the effect of platelet-rich plasma on muscle healing after injury. J Bone Joint Surg Am 2013; 95(11):9808.
25. Li H, Usas A, Poddar M, et al. Platelet Rich Plasma promotes the proliferation of Human Muscle Derived Progenitor Cells and maintains their stemness. PLoS One 2013; 8(6):e64923.
26. Kelc R, Trapecar M, Gradisnik L, Rupnik MS, Vogrin M. Platelet-Rich Plasma, Especially When Combined with a TGF-β Inhibitor Promotes Proliferation, Viability and Myogenic Differentiation of Myoblasts In Vitro. PLoS One 2015; 10(2):e0117302.
27. Delos D, Leineweber MJ, Chaudhury S, Alzoobaee S, Gao Y, Rodeo SA. The effect of PRP on muscle contusion healing in a rat model. Am J Sports Med 2014; 42(9):2067-74.

HISTOLOGICAL FEATURES OF PSEUDOTUMOR AFTER SMALL HEAD DIAMETER METAL-ON-METAL TOTAL HIP ARTHROPLASTY

F. D'ANGELO, A. TESTA, T. BINDA and P. CHERUBINO

Division of Orthopedics and Traumatology, Department of Biotechnologies and Life Sciences, University of Insubria, Varese, Italy

The pathologic aspects of periprosthetic tissues in failed second-generation metal-on-metal (MoM) resurfacing hip arthroplasties have been widely described in terms of necrosis and inflammation. To our knowledge little data are reported on the association of this lesion with the use of small head diameter (<32 mm). In this study we present a small series of pseudo-tumor in small head metal-on-metal total hip arthroplasty focusing our attention on the histologic aspects of the harvested pathologic tissue. The histological examination of our cases showed a presence of lymphocytic infiltrate suggesting a delayed hypersensitivity reaction to metal of type IV (ALVAL) but different to each other in terms of the prevalence of the cellular component. If macrophages are predominant, the pathogenetic mechanism seems to be the reaction against metallic particle. On the other hand, if granulocytes are predominant, it is possible to consider a hypersensitivity reaction. Our observation suggests that the evidence of Pseudotumor in case of small-head metal-on-metal arthroplasty should be considered with the same properties of big-head and therefore these patients should be followed scrupulously.

Second generation metal-on-metal (MoM) hip resurfacing is a recent development in hip arthroplasty. Although early clinical results of second generation MoM hip replacement were encouraging (1, 2, 3, 4, 5), a number of complications have been reported including the formation of inflammatory pseudotumors (6, 7, 8, 9, 10, 11, 12).

Hart et al. (2012) defined Pseudotumor as "sterile, inflammatory lesions within the periprosthetic tissues and have been variously termed masses, cysts, bursae, collections, or aseptic lymphocyte-dominated vasculitis-associated lesions (ALVAL)" (13).

The MoM bearing surface is made from high-carbon, cobalt-chromium-molybdenum alloy. This MoM combination produces less volumetric wear but results in the release of a much larger number of very small (nanometer-sized) particles compared to metal-polyethylene arthroplasties. Analysis of failed first-generation and second-generation MoM resurfacing arthroplasties has led to the identification of a number of histological changes arising from the deposition of these tiny metal particles in peri-implant tissues (14, 15, 16, 17). These include a pronounced macrophage response to wear particles, a perivascular lymphocytic infiltrate, and tissue necrosis. The necrotic and inflammatory changes seen in peri-implant tissues in response to cobalt-chromium (Co-Cr) metal wear debris are thought to be due to either cytotoxicity or to a delayed hypersensitivity reaction (16, 18, 19).

Thus, metal ion measurement becomes a valuable additional tool for diagnosis and patient follow-up. De Pasquale et al. demonstrated that the measurement

Key words: Pseudotumor, small-head, metal-on-metal, hip arthroplasty, Metasul

Mailing address: Prof. Fabio D'Angelo,
Division of Orthopedics and Traumatology,
Department of Biotechnologies and Life Sciences,
University of Insubria, Viale Borri 57,
21100 Varese, Italy
Tel.: +390332278826 Fax:+390332278825
e-mail: fabio.dangelo@uninsubria.it

0393-974X (2015)

of the Co and Cr ions both in whole blood and in serum can be considered a standard test for screening and monitoring patients with MoM since it is strictly correlated with the release of wear particles and corrosion products at the local level (20).

Several authors have reported data on the prevalence of these masses, in both symptomatic and asymptomatic patients after either large-diameter femoral head THA or hip resurfacing arthroplasty, with a large variability of rate (13, 21, 22, 23). Kwon et al. reported a prevalence of 4% in asymptomatic patients undergoing hip resurfacing arthroplasty (23).

To our knowledge few data are reported on the association of this lesion with the use of small head diameter. Gruber FW et al. reported a case report about 2 patients presenting with an extraperitoneal, intrapelvic cystic lesion of the groin due to reaction to metal debris without signs of osteolysis in the plain X-ray or magnetic resonance imaging (MRI) and without typical clinical signs of aseptic component loosening. In both of the described cases, the head diameter was 28 mm (24).

In this study, we have examined the extent of necrosis and inflammation in peri-implant soft tissues, harvested in a series of revised second-generation MoM hip implants with small head, in order to determine the nature and significance of these changes and if they are similar to what happens in >32mm head MoM implants.

MATERIALS AND METHODS

At the author's institution 3 cases of Pseudotumor were revised in 2 patients with small-head (28 mm) metal on metal bearing heads (Metasul bearing total hip®, Zimmer, Warsaw, IN).

The patients were 2 females, with an average age of 63 years (Range 43-73). In all cases the prosthesis was implanted for osteoarthritis due to congenital hip dysplasia. All patients had bilateral THA and one patient has a bilateral Pseudotumor. The average time between primary and revision surgery was 16.2 years (Range 9-19).

The natural history was different in the two patients. The first patient (case n. 1) was referred to our attention for a sudden pain after a minimal trauma in hyperextension of the right hip.

The second patient (case n. 2 and 3) was referred to us after the onset of right iliac vein DVT and the observance of an intrapelvic bilateral mass by gadolinium CT.

The diagnosis of Psuedotumor was suspected at the X-rays (AP pelvis, AP and LAT of the affected side), which showed the classical "bubble sign" together with a metal-on-metal coupling.

In order to confirm the suspicion, the dosage of cobalt-chromium ions serum was made (mean C =1.57 µg/L Co=2,27µg/L; Range C =1–2.4 µg/L; Range Co=0.6–4 µg/L), baseline MRI.

All patients underwent fine needle aspiration under ultrasound guidance with no microorganism growth.

During the revision surgery, performed by the same surgeon (FD) through a posterolateral approach, tissue samples were harvested, fixed in Formaldehyde and prepared for light microscopy observation. All samples were embedded in paraffin wax, sectioned, and stained with Haematoxylin and Eosin and Von Kossa.

RESULTS

The histologic examination showed variability in the amount and distribution of metal residues, the number, type and arrangement of inflammatory cells and the amount of necrotic tissue.

Fig. 1. *Light Microscopy showing the presence of a thin pseudo-capsule, made of connective tissue with cross-linked fibers (10X, E-E staining).*

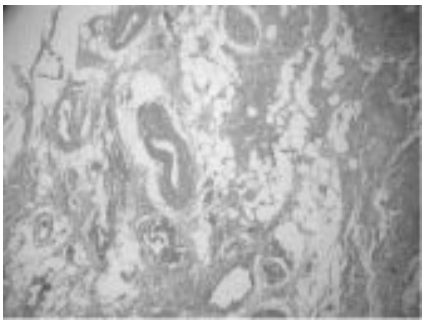


Fig. 2. *New angiogenesis is well represented in the connective layer (25X, E-E staining).*

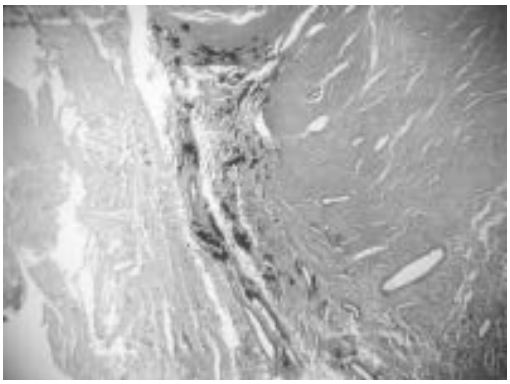


Fig. 3. *Light Microscopy showing aggregates of macrophages and histiocytes with cytoplasmatic metallic inclusion. Also in the extracellular matrix it is possible to observe metallosis. (Case n. 1, X10, E-E staining).*

Fig. 5. *Polarized Light Microscopy did not reveal any birefringent particles, such as Polyethylene ones (X10, E-E staining).*

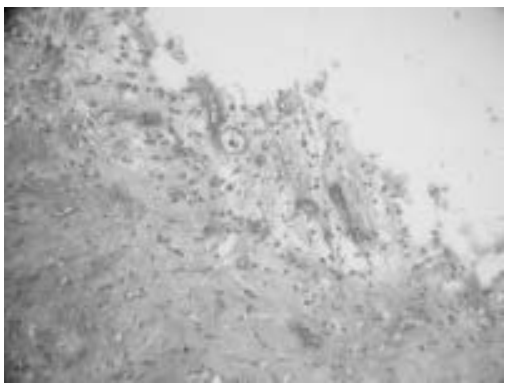


Fig. 4. *Light Microscopy showing the predominance of granulocytes and lymphocytes. No evidence of metallosis even in the matrix. (Case n. 2, X25, E-E staining).*

A common aspect are marginal extensive networks of fibrin where it is possible to observe the migration of fibroblasts in order to create a primitive connective tissue with cross-linked fibres, such a pseudocapsule (Fig. 1). This area is also characterised by the presence of endothelial cells, as evidence of new angiogenesis (Fig. 2).

The cellular component is internally disposed. A general consideration macrophages and lymphocytes were present in all cases, but those with large infiltrates of macrophages presented fewer lymphocyte aggregates as opposed to those that prove dense clusters of lymphocytes, often arranged in the inner layers of the mass. More specifically, in case 1 the prevalent cellular component is represented by macrophages and histiocytes with cytoplasmatic metallic inclusion (Fig. 3), while in both cases 2 and 3 the dominant aspect is the predominance of granulocytes and lymphocytes which is evidence of acute inflammation. In these specimens metallosis was not observed (Fig. 4).

None of the cases reported polarised light microscopy birefringence particles such as polyethylene (Fig. 5).

DISCUSSION

Pseudotumor has been defined in such a broad sense that it has made it difficult for clinicians to thoroughly study it. If the term were more clearly defined with parameters, studies could be done with more focused results. Since it was firstly described, several authors reported different prevalence in metal on metal hip arthroplasty.

A decade ago, a large percentage of hip replacements performed in the United States on virtually all resurfacing arthroplasties used metal-on-metal bearing surfaces. Today however, there has been a major decline in the use of MoM bearings (25, 26). High wear and high concentration of metal debris is related to large-head dimension, in fact, only few reports are present with small-head as in our case.

Although some studies have implied that the wear of the metal is a factor in the failure of some MoM implants, several studies have demonstrated that pseudotumors and other types of complications can

occur even in the absence of high wear (12, 27, 28).

Pandit et al. (12) described 17 cases suggesting that these periprosthetic masses could be a hypersensitive response to a moderate release of particles, as later confirmed by several authors (29, 30).

It's known that the formation of periprosthetic masses is connected to several contributing factors but in these cases described no risk factors are present except for the female sex and the preoperative diagnosis.

The histological examination of our cases showed a presence of lymphocytic infiltrate suggesting a delayed hypersensitivity reaction to the metal of type IV (ALVAL) but conceptually they were different. In case 1 there is a predominance of macrophages embedding metallic particles, thus indicating a response dictated mainly by the excessive production of debris and probably exacerbated by the trauma that decreases the "threshold of accepted debris" (31). Therefore it can be suggested that a combination of necrosis induced by macrophages and hypersensitivity reaction lymphocyte-mediated with a balance, in this case, towards the macrophage response.

This variability reflects the different immunological susceptibility between different patients (27). In confirmation of this theory, the patient has a same implant in contralateral side and there is no sign of failure.

In this case we speculate that necrosis indicates that cytotoxicity is an adjunctive factor. Ultrastructural studies have shown that metal particles phagocytosed by macrophages are transported to lysosomes. High concentrations of metal ions are released in these structures, resulting in apoptosis and cell death with subsequent release of the phagocytized metal (32). The morphology of the necrotic and viable macrophage granulomas seen in pseudotumors and in some cases of MoM component loosening, would be in keeping with this cytotoxic effect causing a vicious circle in which metal wear particle generation leads to macrophage recruitment, particle phagocytosis, apoptosis and cell death with resultant release of metal particles, leading to further macrophage recruitment and repetition of this process.

The situation is different for cases n. 2 and n. 3. In the histological of both masses, we can observe infiltration of lymphocytes and granulocytes while macrophages and plasma are rare, reflecting an acute inflammatory reaction probably due to local response to the prosthetic components.

This analysis seems to support hypersensitivity because eosinophils are typically associated with allergies, are involved in tissue homeostasis, in modulation of Th2 immune response and in the adaptive secreting cytokines and immunoregulatory factors (33, 34). In fact the patient also developed a pseudotumor in contralateral hip (unlike case 1) although obvious component malpositions are not obsereved. The hypersensitivity response to metals is probably variable but in occurrence it could be a major contributory factor to the necrosis and inflammation associated with component loosening and pseudotumor formation.

The CT carried out 18 months later shows reduction of the masses in the abdomen and the patient reported complete resolution of symptoms. The only data in contrast with this theory is the corrosion at the taper, which could explain the high values of Cr-Co detected preoperatively (Cr Co=2.40=4.00). Therefore, also in this case, it may propose a combination of type IV hypersensitivity reaction and necrosis induced by excessive debris, with a preponderance of the former.

CONCLUSION

Total hip arthroplasty is a successful procedure that provides relief from pain and significant improvement in life quality. Despite considerable progress in surgical technique and plant design, some patients develop adverse reactions to metal debris (ARMD), which may result in the formation of pseudotumor and periprosthetic masses characterized by a high local aggressiveness. The use of small-head implant such as Metasul can achieve high clinical-functional results and good long-term survival but are not free from risks.

Our study shows that these lesions could be associated to these implants and that the pathogenesis is multifactorial and not simply associated to wear. The immunoreactivity of each patient plays an important role in the outcome although to which level is not yet known.

Further studies are still necessary in regards to the

materials used, the actual incidence of the disease, its etiology and diagnosis in order to avoid dangerous complications during surgery.

REFERENCES

1. Amstutz HC, Beaulé PE, Dorey FJ, Le Duff MJ, Campbell PA, Gruen TA. Metal-on-Metal Hybrid Surface Arthroplasty: Two to Six-Year Follow-up Study. J Bone Joint Surg 2004; 86:28-39.
2. Treacy RBC, McBryde CW, Pynsent PB. Birmingham hip resurfacing arthroplasty. A minimum follow-up of five years. J Bone Joint Surg 2005; 87:167-70.
3. Back D, Dalziel R, Young D, Shimmin A. Early results of primary Birming- ham hip resurfacings. An independent prospective study of the first 230 hips. J Bone Joint Surg 2005; 87:324-9.
4. Pollard TCB, Baker RP, Eastaugh-Waring SJ, Bannister BC. Treatment of the young active patient with osteoarthritis of the hip; A five- to seven-year comparison of hybrid total hip arthroplasty and metal-on-metal resurfacing. J Bone Joint Surg 2006; 88:592-600.
5. Hing CB, Back DL, Bailey M, Young DA,Dalzier RE, Shimmin AJ. The results of primary Birmingham hip resurfacings at a mean of five years: An independent prospective review of the first 230 hips. J Bone Joint Surg 2007; 89:1431-8.
6. Capello WN, Ireland PH, Trammell TR, Eicher P. Conservative total hip arthroplasty: a procedure to conserve bone stock. Part 1: analysis of sixty- six patients. Part II: analysis of failures. Clin Orthop 1978; (134):59-74.
7. Amstutz HC, Campbell PA, Le Duff MJ. Fracture of the neck of the femur after surface arthroplasty of the hip. J Bone Joint Surg 2004; 86:1874-7.
8. Park YS, Moon YW, Lim SJ, Yang JM, Ahn G, Choi YL. Early osteolysis following second-generation metal-on-metal hip replacement. J Bone Joint Surg 2005; 87:1515-21.
9. Shimmin AJ, Back D. Femoral neck fractures following Birmingham hip resurfacing: a national review of 50 cases. J Bone Joint Surg 2005; 87:463-4.
10. Boardman DR, Middleton FR, Kavanagh TG. A benign psoas mass following metal-on-metal resurfacing of the hip. J Bone Joint Surg 2006; 88:402-4.
11. Jacobs JJ, Hallab NJ. Loosening and osteolysis associated with metal-on- metal bearings. A local effect of metal hypersensitivity? J Bone Joint Surg 2006; 88:1171-2.
12. Pandit H, Glyn-Jones S, McLardy-Smith P, et al. Pseudotumors associated with metal-on-metal hip resurfacings. J Bone Joint Surg 2008; 90:847-51.
13. Hart AJ, Satchithananda K, Liddle AD, et al. Pseudotumors in association with well-functioning metal-on-metal hip prostheses: a case-control study using three-dimensional computed tomography and magnetic resonance imaging. J Bone Joint Surg 2012; 94(4):317-25.
14. Doorn PF, Mirra JM, Campbell PA, Amstutz HC. Tissue reaction to metal on metal total hip prostheses. Clin Orthop 1996; 329(S):187-205.
15. Davis AP, Willert HG, Campbell PA, Learmonth ID, Case CP. An unusual lymphocytic perivascular infiltration in tissues around contemporary metal-on-metal joint replacements. J Bone Joint Surg 2005; 87:18-27.
16. Willert HG, Buchhorn GH, Ing D, Fayyazi A, Flury R, Windler M, Koster G, Lohmann CH. Metal-on-metal bearings and hypersensitivity inpatients with artificial hip joints; A clinical and histomorphological study. J Bone Joint Surg 2005; 87:28-36.
17. Korovessis P, Petsinis G, Repanti M, Repantis T. Metallosis after contemporary metal-on-metal total hip arthroplasty: five to nine-year follow-up. J Bone Joint Surg 2006; 88:1183-91.
18. Hallab N, Merritt K, Jacobs J J. Metal sensitivity in patients with orthopaedic implants. J bone Joint Surg 2001; 83:428-36.
19. Keegan GM, Learmonth ID, Case CP. Orthopaedic metals and their potential toxicity in the arthroplasty patient. A review of current knowledge and future strategies. J Bone Joint Surg 2007; 89:567-73.
20. De Pasquale D, Stea S, Squarzoni S, Bordini B, Amabile M, Catalani S, Apostoli P, Toni A. Metal-on-metal hip prostheses: Correlation between debris in the synovial fluid and levels of cobalt and chromium ions in the bloodstream. Int Orthop. 2014; 38(3):469-75.
21. Kwon YM, Ostlere SJ, McLardy-Smith P,

Athanasou NA, Gill HS, Murray DW. Asymptomatic pseudotumors after metal-on- metal hip resurfacing arthroplasty: prevalence and metal ion study. J Arthroplasty 2011; 26(4):511-8.
22. Williams DH, Greidanus NV, Masri BA, Duncan CP, Garbuz DS. Prevalence of pseudotumor in asymptomatic patients after metal-on-metal hip arthroplasty. J Bone Joint Surg 2011; 93:2164-71.
23. Van der Weegen W, Smolders JM, Sijbesma T, Hoekstra HJ, Brakel K, van Susante JL. High incidence of pseudotumors after hip resurfacing even in low risk patients; results from an intensified MRI screening protocol. Hip Int 2013; 23(3):243-9.
24. Gruber FW, Bock A, Trattnig S, Lintner F, Ritschl P. Cystic lesion of the groin due to metallosis: a rare long-term complication of metal-on-metal total hip arthroplasty. J Arthroplasty 2007; 22(6):923-7.
25. Amstutz HC, Le Duff MJ. Hip resurfacing: a 40-year perspective. HSS J 2012; 8:275-82.
26. Langton DJ, Joyce TJ, Jameson SS, Lord J, Van Orsouw M, Holland JP, Nargol AV, De Smet KA. Adverse reaction to metal debris following hip resurfacing: the influence of component type, orientation and volumetric wear. J Bone Joint Surg Br 2011; 93:164-71.
27. Campbell P, Ebramzadeh E, Nelson S, Takamura K, Smet K, Amstutz H. Histological Features of Pseudotumor-like Tissues From Metal-on-Metal Hips. Clin Orthop Relat Res 2010; 468(9):2321-7.
28. Campbell P, Shimmin A, Walter L, Solomon M. Metal sensitivity as a cause of groin pain in metal-on-metal hip resurfacing. J Arthroplasty 2008; 23:1080-5.
29. Mahendra G, Pandit H, Kliskey K, Murray D, Gill HS, Athanasou N. Necrotic and inflammatory changes in metal-on-metal resurfacing hip arthroplasties. Acta Orthop 2009; 80(6):653-9.
30. Glyn-Jones S, Pandit H, Kwon YM, Doll H, Gill HS, Murray DW. Risk factors for inflammatory pseudotumour formation following hip resurfacing. J Bone Joint Surg Br 2009; 91(12):1566-74.
31. Doorn PF, Campbell PA, Worrall J, Benya PD, McKellop HA, Amstutz HC. Metal wear particle characterization from metal on metal total hip replacements: transmission electron microscopy study of periprosthetic tissues and isolated particles. J Biomed Mater Res 1998; 42(1):103-11.
32. Salvati E, Betts F, Doty S. Particulate metal debris in cemented arthroplasty. Clin Orthop 1993; (293):160-73.
33. Cadossi M, Chiarello E, Savarino L, Mazzotti A, Tedesco G, Greco M, Giannini S. Fast growing pseudotumour in a hairdresser after metal-on-metal hip resurfacing: a case report. Eur Rev Med Pharmacol Sci 2014; 18(1S):29-33.
34. Pandit H, Vlychou M, Whitwell D, Crook D, Luqmani R, Ostlere S, Murray DW, Athanasou NA. Necrotic granulomatous pseudotumors in bilateral resurfacing hip arthoplasties: evidence for a type IV immune response. Virchows Arch 2008; 453(5):529-34.

RELEASE OF GENTAMICIN FROM CEMENT SPACERS IN TWO-STAGE PROCEDURES FOR HIP AND KNEE PROSTHETIC INFECTION: AN I*N VIVO* PHARMACOKINETIC STUDY WITH CLINICAL FOLLOW-UP

G. BALATO[1], T. ASCIONE [2], D. ROSA[1], P. PAGLIANO[2], G. SOLARINO[3], B. MORETTI[3] and M. MARICONDA[1]

[1]*Department of Orthopaedic Surgery, School of Medicine, Federico II University, Naples, Italy;*
[2]*Department of Infectious Diseases, D. Cotugno Hospital, AORN Dei Colli, Naples, Italy;*
[3]*Department of Orthopaedic Surgery, School of Medicine, Aldo Moro University, Bari*

Eighteen patients undergoing two-stage exchange arthroplasty for infected total hip or knee arthroplasty using gentamicin-loaded bone cement spacers (80g bone cement, 2 g gentamicin and 2 g clindamycin) were studied. The concentration of gentamicin eluted from the spacers was assessed on samples of blood, urine, and drainage fluid that were collected from each patient at set intervals during the 48 hours following the first-stage surgery. The hip and knee cement spacers showed similar curve of release over the first postoperative hours (early peak followed by slow release), but the mean gentamicin concentration in the drainage fluid was higher in patients with hip spacers compared to patients with knee spacers (30.61 ± 19.47 mg/L vs. 17.43 ± 13,63 mg/L, p<0.05). In patients with hip spacers, the mean, maximum, and minimum concentration of gentamicin was higher with respect to the minimum inhibitory concentration (MIC) break point for *Staphylococcus* spp, *Pseudomonas Aeruginosa* and Enterobacteriaceae throughout the first postoperative 48 h. Conversely, in 25% of patients with a knee spacer a drug concentration below the MIC break point for Gram negative bacteria was found in the drainage fluid after 12 h. Gentamicin levels in the blood samples were negligible over the entire time interval and were steadily well below the renal toxicity reference. The highest urinary concentration of gentamicin was observed between 4 and 9 h postoperatively. Subsequently, it gradually declined until 48 h. Clinically, the rate of cure was 100% at a mean follow-up of 113 weeks (range 90-182). Gentamicin-loaded cement spacers offer the advantage of achieving early high concentrations of the antibiotic directly at the site of infection but especially in the knee a systemic antibiotic therapy must be given as a complement to the spacer implantation to eradicate periprosthetic joint infection (PJI).

Periprosthetic joint infection (PJI) is a rare yet devastating complication after total joint replacement. The overall prevalence of PJI ranges between 0.5% and 3%, but the risk for infection is higher for total knee arthroplasty (TKA) than total hip artroplasty (THA) (1-4). Two-stage revision surgery with antibiotic-loaded spacer implantation represents the standard of care for patients who develop chronic infection at the site of a total joint replacement (5-8). The pharmacokinetic properties of antibiotic-loaded bone-cement spacers

Key Words: total hip arthroplasty, total knee arthroplasty, infection, antibiotic-loaded cement spacer, gentamicin, in vivo study

Mailing address: Giovanni Balato MD,
Department of Orthopaedic Surgery, School of Medicine,
Federico II University,
Via Pansini 5,
Naples (NA), Italy,
Tel.: +390817462854 Fax: +390817463895
e-mail: giovannibalato@gmail.com

0393-974X (2015)

implanted during first-stage surgery have been thoroughly investigated *in vitro*, but there is a paucity of *in vivo* data on the antibiotic release from these devices (9-13). Some studies suggested that the elution of antibiotics from cement spacers could help to set the ideal moment for the explantation of cement and the prosthetic reimplantation (12-14). Nevertheless, differences in methods and experimental conditions have complicated the attempts to draw definite conclusions from the available studies. Moreover, a number of questions including effective concentration and the inhibitory ability of the antibiotic released at the site of implantation of the cement spacer, as well as the duration of prophylactic effect, still remains unanswered. The aims of this study were: 1) to determine the concentration of gentamicin eluted from antibiotic-loaded cement spacers in samples of blood, urine, and drainage fluid of patients who had undergone the first surgical stage of two-stage revision procedures for PJI; 2) to assess the local effective inhibitory activity of the antibiotic released from the spacers; 3) to evaluate the clinical outcome of PJI in these patients.

MATERIALS AND METHODS

Patients

All patients who were referred to our institution for suspected PJI between January 2012 and November 2013 were considered for this study. Only patients with a diagnosis of delayed PJI sustained by gentamicin susceptible bacteria undergoing two-stage revision surgery with implantation of a gentamicin-loaded cement spacer were included. The exclusion criteria were: 1) acute PJI; 2) infection sustained by gentamicin resistant bacteria; 3) severe life-threatening comorbidities that contraindicated surgery; 4) simultaneous intake of gentamicin; 5) known or suspected allergy to aminoglycosides; 6) follow-up of less than one year. The research was conducted in accordance with the Declaration of Helsinki and the national and institutional ethical research standards. All patients gave their informed consent prior to being included in the study. PJI was diagnosed according to the criteria of the Musculoskeletal Infection Society (15). A total of 18 patients with PJI fulfilled the inclusion and exclusion criteria (Table I). As shown in Table I, most infecting microorganisms were Gram positive. The microbiological diagnosis was obtained preoperatively through cultures for aerobic and anaerobic bacteria on synovial fluid in 13/18 patients (72%) or sinus tract in 5/18 patients (28%).

Surgical technique

All patients underwent two-stage revision of their infected arthroplasty. At the time of the first surgical stage, removal of the primary implant and bone cement along with thorough debridement of all infected tissue and bone was carried out. Subsequently, a molded antibiotic-

Table I. *Characteristics of patients.*

Patients	N = 18
Age, years	
Mean ± SD	65 ± 11.5
Range	51-77
Gender	
Male	6 (34%)
Female	12 (67%)
Type of prosthesis	
THA	10 (56%)
TKA	8 (44%)
Bacterial cultures	
MSSA	3 (16,7%)
MRSA	5 (27,8%)
CoNS *Staphylococcus*	5 (27,8%)
Pseudomonas aeruginosa	2 (11%)
Enterobacteriaceae	3 (16.7%)

***SD**: Standard deviation; **THA**: Total Hip Arthroplasty; **TKA**: Total Knee Arthroplasty; **MSSA**: Methicillin-sensible-Staphylococcus aureus; **MRSA**: Methicillin-resistant- Staphylococcus aureus; **CoNS**: Coagulase negative*

loaded cement spacer (80g bone cement, 2 g gentamicin, and 2 g clindamycin) (Refobacin Revision Biomet) was inserted in the knee and hip joint cavity. A subfascial drain was finally left in the joint and kept in place for 48 h postoperatively. Before surgery, IV vancomycin (Vancocin, 1g, twice daily) was administered to each patient. After surgery, all patients were given parenteral antibiotics according to the pathogen identification. The mean interval between first and second surgical stage was 11±0.83 weeks (range 10-12). The second surgical stage was performed after clinical and laboratory findings had normalized. Antibiotic therapy was discontinued at least two weeks before surgery.

Antibiotic determination

Specimens of blood, urine, and drainage fluid were sequentially collected at set intervals for 48 h after following the first-stage surgery. Postoperatively, drainage fluid samples were collected at 1, 2, 3, 4, 6, 9, 12, 15, 18, 21, 24, 30, 36, 42 and 48 h, venous blood samples at 1, 2, 3, 4, 24 and 48 h and urine samples at 1, 2, 2, 3, 6, 9, 12, 24 and 48 h.

At the time of prosthetic reimplantation, a sample of synovial fluid was also obtained to evaluate the presence and concentration of gentamicin. In each patient, a fluorescence polarization immunoassay (Abbot, Wiesbaden, Germany) was used to determine the concentration of gentamicin in the samples of drainage fluid, peripheral blood and urine. The lowest measurable concentration of gentamicin in these fluids was 0.22 mg/L. Concentrations beyond the upper limit were diluted in sterile water and multiplied by the dilution coefficients to calculate the final concentration. Maximum drug concentrations (Cmax) were calculated from the concentration-time curve for gentamicin release in the joint cavity. The local antibiotic concentration was compared to the minimum inhibitory concentration (MIC) (EUCAST) break point for *Staphylococcus* spp, *Pseudomonas Aeruginosa* and Enterobacteriaceae (16). The area under the curve for the antibiotic concentratiom from 0 to 48 h ($AUC_{0–48}$) was calculated with the linear trapezoid rule. For each patient three pharmacokinetic (PK)/pharmacodynamics (PD) parameters, including the ratio of Cmax to the MIC, the duration of sufficient antibiotic release ($T_{>MIC}$) (h), and the ratio of $AUC_{0–48}$ to the MIC were calculated to assess differences in the antibacterial inhibitory activity between hip and knee spacers. Blood concentrations of gentamicin were compared to the renal toxic concentration (2 mg /L).

Follow-up

C reactive protein (CRP), erythrocyte sedimentation rate (ESR) and a full blood count were assessed every 4 days during the 2-week period of intravenous antibiotic treatment. Subsequently, routine laboratory data including full blood count, ESR, CRP, liver enzymes, blood urea, creatinine, and a radiographic examination (anteroposterior and lateral views) of the joint were obtained every month thereafter. Cure was defined by the normalization of clinical, laboratory and radiologic findings at least one year after the discontinuation of antibiotic treatment.

Statistical analysis

Continuous variables were expressed as mean ± standard deviation (SD). An unpaired Student t test was used to assess differences between two means. Significance was set at $p<0.05$. SPSS software (SPSS Inc., Chicago, Illinois, USA) was used for the database and statistics.

RESULTS

Curves of antibiotic release in the drainage fluid showed a transient initial peak followed by slow release (Fig. 1). Both hip and knee PMMA spacers showed a similar release pattern, but the mean gentamicin concentration at the first postoperative hour was higher in those patients with hip spacers compared to the patients with knee spacers (30.61±19.47 mg/L *vs* 17.43±13,63 mg/L, $p<0.05$).

The maximum drug concentration was reached in the first hour after surgery, being 53.9 mg/L and 44,1 mg/L, respectively, for hip and knee spacers ($p<0.05$). The minimum concentration was detected 48 h postoperatively, with a mean value of 14.6 mg/L and 8.7 mg/L for knee and hip spacers, respectively (p=NS). The mean concentration of antibiotics released from the spacers at all time intervals is reported in Table II.

The mean, maximum and minimum concentration of gentamicin was steadily higher in comparison with the MIC break point for *Staphylococcus spp*, *Pseudomonas Aeruginosa* and Enterobacteriaceae throughout the first 48 postoperative h in patients with hip spacers (Fig. 2). Conversely, the minimum drug concentration fell below the MIC break point for Gram-negative bacteria in the knee drainage fluid between 12 and 30 h in 25% of patients, and between 36 and 48 h in 50% of patients (Fig. 3). The results of the three PK/PD parameters are showed in Table III.

Hip spacers showed significantly greater and

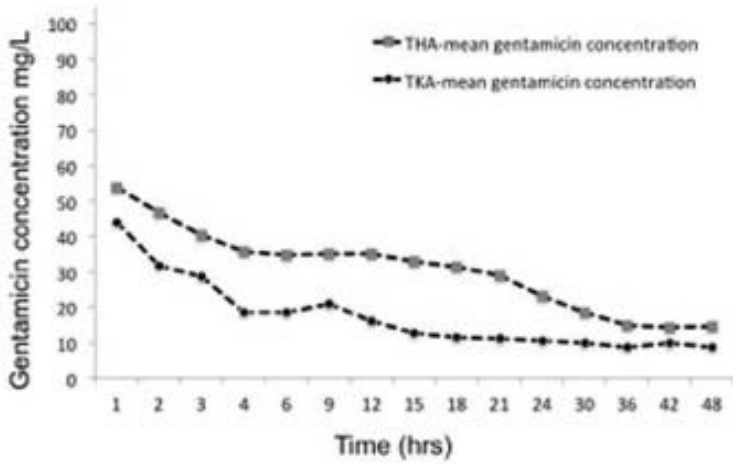


Fig. 1. *Mean gentamicin concentration (mg/L) in the hip and knee drainage fluid.*

Table II. *Mean concentration of gentamicin (mg/L) released from the spacers at all time intervals.*

Time	Hip spacer	Knee spacer	p-value
1 hour	53.9	44.1	0.216
2 hour	46.8	31.6	0.196
3 hour	39.3	28.7	0.569
4 hour	35.6	18.4	0.010
6 hour	34.7	18.6	0.009
9 hour	35.1	20.9	0.913
12 hour	35.0	16.0	0.038
15 hour	32.9	12.8	0.019
18 hour	31.4	11.4	0.014
21 hour	28.8	11.2	0.020
24 hour	23.0	10.5	0.025
30 hour	18.5	9.9	0.057
36 hour	14.9	8.8	0.056
42 hour	14.3	9.8	0.08
48 hour	14.6	8.7	0.180

more prolonged antibacterial activity as compared to knee spacers. No dosable antibiotic in the synovial fluid of any patients was detected at the time of the implantation of revision arthroplasty. Serum gentamicin levels were negligible over the entire time interval. They were steadily well below the renal toxicity reference (Fig. 4). The highest urinary excretion of gentamicin was observed between 4 and 9 h postoperatively. Subsequently, it gradually declined until 48 h (Fig. 5). Clinically, the rate of cure was 100% at a mean follow-up of 113 weeks (range 90-182). No cases of renal side effects throughout

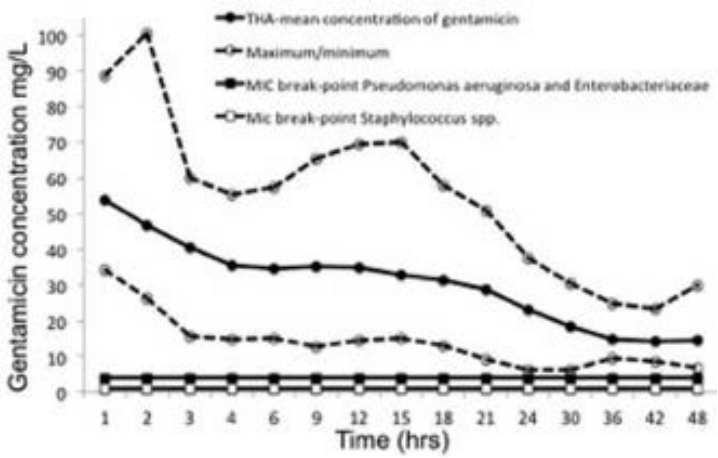


Fig 2. *Comparison of mean, maximum, and minimum gentamicin concentration (mg/L) in the drainage fluid to the MIC break-point for Staphylococcus spp, Pseudomonas Aeruginosa, and Enterobacteriaceae in patients with hip spacer.*

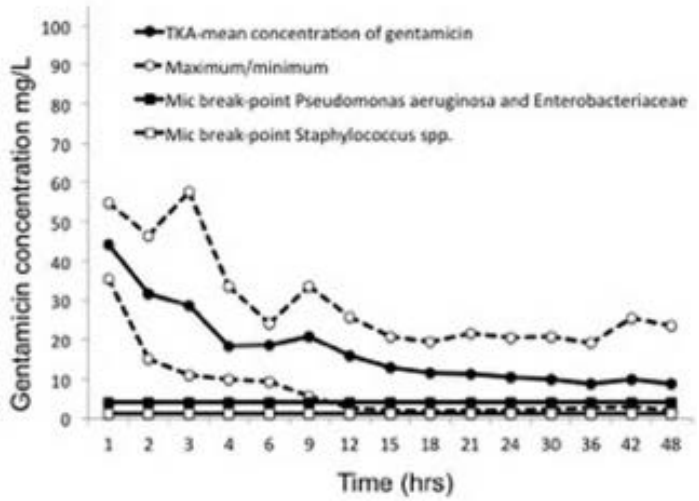


Fig. 3. *Comparison of mean, maximum, and minimum gentamicin concentration (mg/L) in the drainage fluid to the MIC break-point for Staphylococcus spp, Pseudomonas Aeruginosa, and Enterobacteriaceae in patients with knee spacer.*

Table III. *Pharmacokinetic and pharmacodynamic parameters (mean ± standard deviation).*

	AUC/MIC *Staphylococcus spp.*	AUC/MIC Gram negative bacteria	Cmax/MIC *Staphylococcus spp.*	Cmax/MIC Gram negative bacteria	T > MIC *Staphylococcus spp.* (hours)	T > MIC Gram negative bacteria (hours)
Hip spacer	1171,5 ±536,7	292,86 ± 134,2	53,5 ±26,3	13,34 ±6,5	48	48
Knee spacer	618,4 ± 381,7	154 ±95,4	47,2 ±9,8	11,74 ±2,5	48	33,7 ±17,2
p value	<0,05	<0,05	ns	ns	ns	<0,05

***AUC**: area under the curve; **MIC**: minimum inhibitory concentration; $\boldsymbol{C_{max}}$: maximum drug concentration; **T**: time.*

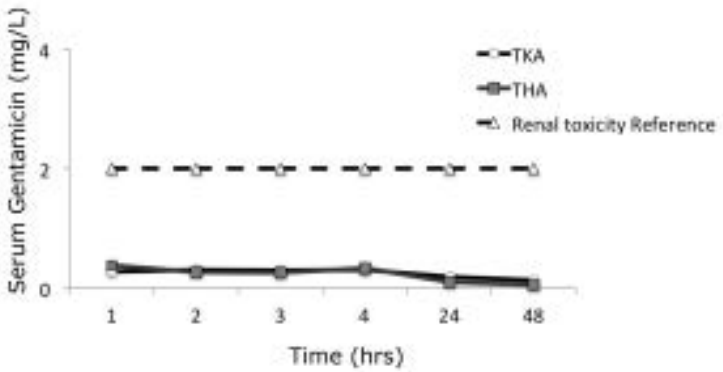


Fig. 4. *Mean serum gentamicin concentration (mg/L) in patients with hip and knee spacers and renal toxicity reference.*

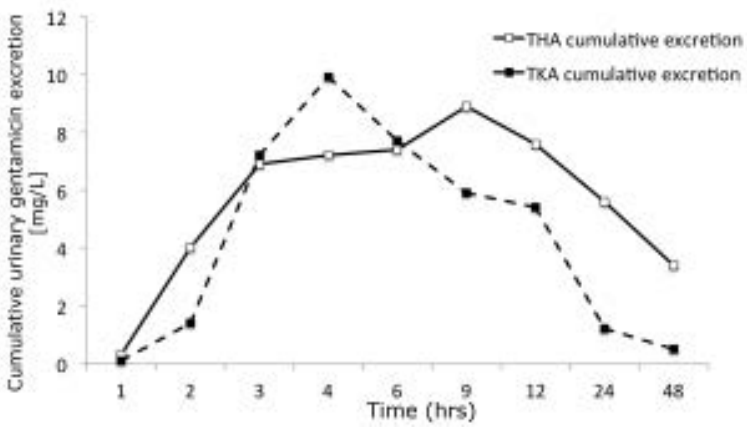


Fig. 5. *Cumulative urinary gentamicin excretion (mg/L).*

the entire follow-up interval were diagnosed.

DISCUSSION

Two-stage revision arthroplasty is a well established method for the care of patients with chronic PJI (5-8). In the current study, this procedure, in combination with thorough debridement and the systemic administration of selective antibiotics, was proven highly effective in eradicating the infection after TKA or THA. In two-stage revision procedures, antibiotic-loaded cement spacers are used during the interim period to maintain joint cavity thereby facilitating the reimplantation. Their use is also intended to deliver antibiotics locally contributing to the cure of the infection. Since their introduction by Buchholz and Engelbrecht (17), antibiotic-impregnated bone cements have been widely used as a delivery vehicle for the local administration of antibiotics in infected joints as they can release higher concentrations of antibiotics compared to the systemic administration of these drugs (11).

The selection of the antibiotic in the cement spacer is crucial to increase the chance of eradicating the PJI, especially in the setting of biofilm formation. However, despite the widespread use of antibiotic-loaded cement, a number of questions about their antibacterial activity *in vivo* still remains to be

addressed.

Indeed, the therapeutic level and the actual inhibitory activity of antibiotic at the site of implantation in the early post-operative period as well as the duration of antimicrobial effect are still matter of debate. This *in vivo* study showed that the release of gentamicin from knee and hip spacers is characterized by a transient early peak followed by slow release. Although the hip and knee spacers have similar pattern of antibiotic release over the first 48 postoperative h, the mean gentamicin concentration is higher in patients with hip spacer than in patients with knee spacer.

Since the bactericidal activity of aminoglycosides is concentration-dependent (20), this is a crucial result in relation to the clinical outcome of two-stage knee revisions. The different local concentration of gentamicin released from the hip and knee spacers can be likely ascribed to different surface area and the bone/device contact area (12). Indeed, previous *in vivo* studies reported positive correlation between the surface area of vancomycin-loaded spacers and their elution characteristics (21, 22) thereby suggesting that increasing the surface-to-volume ratio may enhance the antibiotic release.

We also found that the concentration of gentamicin in the hip joint fluid is higher than the MIC breakpoint of the Gram positive and Gram-negative bacteria under examination. By contrast, in the knee joint fluid, sub-inhibitory concentrations of gentamicin against *Pseudomonas aeruginosa* and Enterobacteriaceae were detected nine hours after surgery. The formation and maturation of biofilm on orthopaedic implants occurs in the early postoperative hours (23), and the lack of an effective concentration of antibiotic in the knee joint cavity may represent a disadvantage for treating PJIs. Indeed, bacteria may survive on antibiotic-loaded cement and even develop antibiotic resistance when exposed to prolonged sub-inhibitory concentration of antibiotics (24-27).

The survival of bacteria on cement spacers, irrespective of the fact that they may have been preoperatively susceptible to the antibiotics included in the spacer may eventually result in a clinical reinfection or a deep postoperative infection (26). However, one previous *in vitro* study suggested that different bone cements seem to have a different "window-of-effectiveness" with regard to the reduction in biofilm formation that did not relate with the gentamicin-release kinetics (28).

As far as the duration of the effect is concerned, some studies demonstrated that the antibiotic effectiveness of cement spacers could last from several days to several months after surgery (30-33). For instance, an *in vivo* prospective study reported that in most patients effective antibiotic levels are maintained for at least 4 months following implantation, particularly when a minimum 3.6 g of tobramycin is added to a 40 g package of bone cement (11). In the present study, both knee and hip spacers failed to show a long time antibiotic elution**,** as evidenced by the absence of dosable gentamicin in joint cavities at the time of revision surgery. Nevertheless, we obtained a 100% infection eradication rate. This result emphasizes the importance of a thorough debridement at the time of the first surgical stage and supports the prolonged administration of systemic antibiotics between the two surgical stages. Regarding the tolerability and safety of antibiotic-loaded cement spacers, the treatment was well tolerated in all patients in this series and no serious adverse reactions were observed.

This result is in keeping with previous studies stating the safety of antibiotic-impregnated e bone cements (34-43). In fact, low rates (0-10%) of renal damage have been reported after spacer implantation (19).

Our study has two main shortcomings. First, it suffers from a lack of comparison of results obtained in patients treated with antibiotic-loaded cement spacers and controls (i.e. patients treated without spacer or with pharmacologically inactive cement spacers). A study design like this prevented us to draw definite conclusions on the therapeutic role of locally delivered *vs* systemically administered antibiotics. However, it would have been unethical to deny the use of systemic antibiotics in one of the experimental groups.

Nevertheless, this study clearly demonstrates that a bactericide concentration of gentamicin delivered by the spacers is still found in the hip joint 48 h after implantation whereas concerns about the concentration of antibiotic in the knee joint cavity exist. This result emphasizes the importance of

systemic antibiotics, given as a complement to the spacer implantation. The second shortcoming is the limited number of patients enrolled. Conversely, the main strenght of the present study is its assessment of the *in vivo* postoperative elution of gentamicin in the light of clinical results.

In conclusion, gentamicin-loaded cement hip spacers offer the advantage of achieving early high concentrations of the antibiotic directly at the site of infection with negligible systemic absorption and adverse effects. Locally released gentamicin may contribute to the cure, but the simultaneous and prolonged systemic administration of specific antibiotics is mandatory for the management of PJIs, particularly after TKA.

REFERENCES

1. Hanssen AD, Rand JA. Evaluation and treatment of infection at the site of a total hip or knee arthroplasty. Instr Course Lect 1999; 48:111-22.
2. Joseph TN, Chen AL, Di Cesare PE. Use of antibiotic-impregnated cement in total joint arthroplasty. J Am Acad Orthop Surg 2003; 11:38-47.
3. Peersman G, Laskin R, Davis J, Peterson M. Infection in total knee replacement: a retrospective review of 6489 total knee replacements. Clin Orthop Relat Res 2001; 392:15-23.
4. Durbhakula SM, Czajka J, Fuchs MD, Uhl RL. Spacer endoprosthesis for the treatment of infected total hip arthroplasty. J Arthroplasty 2004; 19:760-7.
5. Leone JM, Hanssen AD. Management of infection at the site of a total knee arthroplasty. J Bone Joint Surg Am 2005; 87:2335-48.
6. Clyburn TA, Cui Q. Antibiotic laden cement: current state of the art. AAOS Now 2007; 1:17-18.
7. Cui Q, Mihalko WM, Shields JS, Ries M, Saleh KJ. Antibiotic impregnated cement spacers for the treatment of infection associated with total hip or knee arthroplasty. J Bone Joint Surg Am 2007; 89:871-82.
8. Jacofsky DJ, Hanssen AD. The infected total knee arthroplasty. In Hip and knee reconstruction 3. 3rd Edition. R.L. Barrack, et al, eds. OKU, Orthopaedic knowledge update. Rosemont 2006; p. 177.
9. Anagnostakos K, Wilmes P, Schmitt E, Kelm J. Elution of gentamicin and vancomycin from polymethylmethacrylate beads and hip spacers in vivo. Acta Orthop 2009; 80:193-7.
10. Sterling GJ, Crawford S, Potter JH, Koerbin G, Crawford R. The pharmacokinetics of Simplex-tobramycin bone cement. J Bone Joint Surg Br 2003; 85:646-9.
11. Masri BA, Duncan CP, Beauchamp CP. Long-term elution of antibiotics from bone-cement: an in vivo study using the prosthesis of antibiotic-loaded acrylic cement (PROSTALAC) system. J Arthroplasty 1998; 13:331-8.
12. Bertazzoni Minelli E, Benini A, Samaila E, Bondi M, Magnan B. Antimicrobial activity of gentamicin and vancomycin combination in joint fluids after antibiotic-loaded cement spacer implantation in two-stage revision surgery. J Chemother 2015; 27:17-24.
13. Mutimer J, Gillespie G, Lovering AM, Porteous AJ. Measurements of *in vivo* intra-articular gentamicin levels from antibiotic loaded articulating spacers in revision total knee replacement. Knee 2009; 16:39-41.
14. Anagnostakos K, Fürst O, Kelm J. Antibiotic-impregnated PMMA hip spacers: Current status. Acta Orthop 2006; 77:628-37.
15. Parvizi J, Zmistowski B, Berbari EF, et al. New definition for periprosthetic joint infection: from the Workgroup of the Musculoskeletal Infection Society. Clin Orthop Relat Res 2011; 469:2992-4.
16. EUCAST Disk Diffusion Test for Routine Antimicrobial Susceptibility Testing. Available from: http://www.eucast.org/clinical_breakpoints. Last accessed (text modified) 01 January 2015.
17. Buchholz H W, Engelbrecht H. Depot effects of various antibiotics mixed with Palacos resins. Chirurg 1970; 41:511-5.
18. Parvizi J, Gehrke T, Chen AF. Proceedings of the International Consensus Meeting on Periprosthetic Joint Infection. Bone Joint J 2013; 95:1450-2.
19. Iarikov D, Demian H, Rubin D, Alexander J, Nambiar S. Choice and doses of antibacterial agents for cement spacers in treatment of prosthetic joint infections: review of published studies. Clin Infect Dis 2012; 55:1474-80.
20. McLean AJ, Ioannides Demos LL, Li SC, Bastone EB, Spicer WJ. Bactericidal effect of gentamicin peak concentration provides a rationale for admin-

istration of bolus doses. J Antimicrob Chemother 1993; 32:301-5.
21. Greene N, Holtom PD, Warren CA, Ressler RL, Shepherd L, McPherson EJ, Patzakis MJ. *In vitro* elution of tobramycin and vancomycin polymethylmethacrylate beads and spacers from Simplex and Palacos. Am J Orthop 1998; 27:201-5.
22. Holtom PD, Warren CA, Greene NW, Bravos PD, Ressler RL, Shepherd L, McPherson EJ, Patzakis MJ. Relation of surface area to in vitro elution characteristics of vancomycin-impregnated polymethylmethacrylate spacers. Am J Orthop (Belle Mead NJ) 1998; 27:207-10.
23. Gristina AG, Shibata Y, Giridhar G, Kreger A, Myrvik QN. The glycocalyx, biofilm, microbes, and resistant infection. Semin Arthroplasty 1994; 5:160-70.
24. Anagnostakos K, Hitzler P, Pape D, Kohn D, Kelm J. Persistence of bacterial growth on antibiotic-loaded beads: is it actually a problem? Acta Orthop 2008; 79:302-7.
25. Anagnostakos K, Kelm J. Review Enhancement of antibiotic elution from acrylic bone cement. J Biomed Mater Res B Appl Biomater 2009; 90:467-75.
26. Corona PS, Espinal L, Rodríguez-Pardo D, Pigrau C, Larrosa N, Flores X. Antibiotic susceptibility in gram-positive chronic joint arthroplasty infections: increased aminoglycoside resistance rate in patients with prior aminoglycoside-impregnated cement spacer use. J Arthroplasty 2014; 29:1617-21.
27. Neut D, Hendriks JG, van Horn JR, van der Mei HC, Busscher HJ Pseudomonas aeruginosa biofilm formation and slime excretion on antibiotic-loaded bone cement. Acta Orthop 2005; 76:109-14.
28. van de Belt H, Neut D, Schenk W, van Horn JR, van Der Mei HC, Busscher HJ. Staphylococcus aureus biofilm formation on different gentamicin-loaded polymethylmethacrylate bone cements. Biomaterials 2001; 22:1607-11.
29. Hanssen AD. Prophylactic use of antibiotic bone cement. An emerging standard–in opposition. J Arthroplasty 2004; 19:73-7.
30. Hoff SF, Fitzgerald RHJ, Kelly PJ: The depot administration of penicillin G and gentamicin in acrylic bone cement. J Bone Joint Surg 1981; 63-A:798-804.
31. Kuechle DK, Landon GC, Musher DM, Noble PC. Elution of vancomycin, daptomycin, and amikacin from acrylic bone cement. Clin Orthop 1991; 264:302-8.
32. Goodell JA, Flick AB, Herbert JC, Howe JG. Preparation and release characteristics of tobramycin-impregnated polymethylmethacrylate beads. Am J Hosp Pharm 1986; 43:1454-61.
33. Bunetel L, Segui A, Langlais F, Cormier M: Osseous concentrations of gentamicin after implantation of acrylic bone cement in sheep femora. Eur J Drug Metab Pharmacokinet 1994; 19:99-105.
34. Cabrita HB, Croci AT, Camargo OP, Lima AL. Prospective study of the treatment of infected hip arthroplasties with or without the use of an antibiotic-loaded cement spacer. Clinics (Sao Paulo) 2007; 62:99-108.
35. Whittaker JP, Warren RE, Jones RS, Gregson PA. Is prolonged systemic antibiotic treatment essential in two-stage revision hip replacement for chronic Gram-positive infection? J Bone Joint Surg Br 2009; 91:44-51.
36. Evans RP. Successful treatment of total hip and knee infection with articulating antibiotic components: a modified treatment method. Clin Orthop Relat Res 2004; 427:37-46.
37. Hofmann AA, Goldberg T, Tanner AM, Kurtin SM. Treatment of infected total knee arthroplasty using an articulating spacer: 2- to 12-year experience. Clin Orthop Relat Res 2005; 430:125-31.
38. Springer BD, Lee GC, Osmon D, Haidukewych GJ, Hanssen AD, Jacofsky DJ. Systemic safety of high-dose antibiotic-loaded cement spacers after resection of an infected total knee arthroplasty. Clin Orthop Relat Res 2004; 427:47-51.
39. Hsieh PH, Huang KC, Tai CL. Liquid gentamicin in bone cement spacers: in vivo antibiotic release and systemic safety in two-stage revision of infected hip arthroplasty. J Trauma 2009; 66:804-8.
40. Gooding CR, Masri BA, Duncan CP, Greidanus NV, Garbuz DS. Durable infection control and function with the PROSTALAC spacer in two-stage revision for infected knee arthroplasty. Clin Orthop Relat Res 2011; 469:985-93.
41. Jung J, Schmid NV, Kelm J, Schmitt E, Anagnostakos K. Complications after spacer implantation in the treatment of hip joint infections. Int J Med Sci 2009; 6:265-73.

42. Isiklar ZU, Demirors H, Akpinar S, Tandogan RN, Alparslan M. Two-stage treatment of chronic staphylococcal orthopaedic implantrelated infections using vancomycin impregnated PMMA spacer and rifampin containing antibiotic protocol. Bull Hosp Jt Dis 1999; 58:79-85.

43. Koo KH, Yang JW, Cho SH, et al. Impregnation of vancomycin, gentamicin, and cefotaxime in a cement spacer for two-stage cementless reconstruction in infected total hip arthroplasty. J Arthroplasty 2001; 16:882-92.

PATHOLOGIC AND IMPENDING FRACTURES: BIOLOGICAL AND CLINICAL ASPECTS

M.S. SPINELLI [1], S. CAMPI[2], F.M. SACCHETTI[3], B. ROSSI [4], A. DI MARTINO[5], S. GIANNINI[6] and A. PICCIOLI[7]

[1]Department of Orthopaedics and Trauma Surgery, Fatebenefratelli Hospital, Rome, Italy; [2]Nuffield Department of Orthopaedics, Rheumatology and Musculoskeletal Sciences, University of Oxford, United Kingdom; [3]Department of Orthopaedics and Trauma Surgery, "Cristo Re" Hospital, Rome, Italy; [4]Department of Orthopaedics and Trauma Surgery, Alto Chiascio Hospital, Gubbio-Gualdo Tadino; [5]CIR, Center of Integrated Research, Department of Orthopaedics and Trauma Surgery, University Campus Bio-Medico of Rome, Italy; [6]Department of Radiology of "Villa Stuart Sport Clinic", "Foro Italico" University of Rome, Italy; [7]Oncologic Center "Palazzo Baleani" Azienda Policlinico "Umberto I", Rome, Italy

Bone metastases from carcinomas are epidemiologically rising because of the increased survival rate of oncologic patients, related to several factors such as improvement of primary and secondary screening, advancement of medical research and technology and the better understanding of mechanisms underlying bone metastases origination from primary tumor. Skeletal Related Events (SREs) can seriously affect quality of life in patients with metastatic disease. These events include the necessity of radiotherapy or bone surgery, malignant hypercalcemia, pathologic fractures and spinal cord compression. Among the SREs, pathologic fractures are the most disabling events and represent an emergency in these delicate patients. A pathologic fracture is defined as a fracture that occurs at the level of a pre-existing bone lesion (that is often a tumor), spontaneously or as the result of low-energy trauma (1). The pre-existence of the metastatic lesion in the bone, its evaluation and the assessment of progression can make these complications predictable and preventable. Pathologic fractures imply several severe consequences, including patient immobilization (in the case of fractures involving the lower limbs), loss of autonomy, anaemia, need of blood transfusion, discontinuation of medical therapies or radiotherapy and protracted hospitalization. Secondary effects of prolonged immobilization and loss of autonomy further lengthen this list of complications in patients who are already significantly limited in their activities. In the present paper, the authors present a review on the main aspects involved in bone metastastic disease: biology, quality of life, economic impact and survival.

Biology of bone metastasis: the metastatic niche

The "metastatic niche" is a microenvironment described as a combination of cancer cells, bone marrow cells, bone matrix and signalling molecules. The bone microenvironment is comprised of mineralized bone matrix and many cell types: osteoblasts, osteoclasts, osteocytes, mesenchymal stem/stromal cells (MSCs), endothelia, immune cells and hematopoietic stem cells (HSPCs). All bone stromal cells with production of trophic factors,

Key words: Bone metastasis, impending fracture, pathologic fracture

Mailing Address:
M.Silvia Spinelli, MD,
P.zza Fatebenefratelli 2,
00186 Rome, Italy
tel.: +39 06 6837241
e-mail: msilviaspinelli@yahoo.it

0393-974X (2015)

cytokines and chemokines promotes homing, adhesion and hibernation or proliferation of bone metastatic cells. These cells, originated from the bone marrow, have recently attracted attention of researchers because they are genetically stable hence representing a new target in cancer treatment (2).

Disseminated tumor cells colonise areas with red bone marrow because they need active hematopoietic process for nutrition and growth. Only a very small rate of tumor cells escape from the primary tumors, disseminating and homing to the secondary organs. One essential pathway governing cell homing is the CXCL12–CXC-chemokine receptor 4 (CXCR4) axis. CXCL12 is produced by MSCs, endothelial cells and osteoblasts. After homing the metastatic cells, survived at immune cells, adhered to the stroma with multiple adhesion molecules. Integrin αvβ3 promotes their adherence to stroma components such as osteopontin, fibronectin, vitronectin, and thrombospondin. Cancer cells also express α4β1 integrin to engage to intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expressed by stromal and vascular cells (3). After adhesion metastatic cancer cells disturb the balanced activity of osteoclasts and osteoblasts and move the equilibrium to bone lysis or bone growth. The osteolytic bone metastasis of breast cancer has been frequently cited as the classic example of "seed and soil" interactions between tumor and stroma.

The foundation of this model proposed by Stephen Paget in Lancet suggested that cancer cells scatter throughout the body as pre-metastatic "seeds" which grow best when they encounter a specific fertile "soil". "Soil" is represented by bone in the case of breast tumors (4). This model has been expanded in the molecular era to the "vicious cycle" model (5) in which bone enhances tumor activity and in turn stimulates the bone environment to be more fertile for tumor cells implantation. Crosstalk between tumor cells and the microenvironment acts as a vicious cycle of both tumor growth and bone remodeling.

Growth factors secreted by bone cells induce tumor cell production of tumor necrosis factor alpha (TNF-α), IL11, VCAM-1, MMP1, Jagged 1 and parathyroid hormone-related protein (PTHrP), fibroblast growth factor (FGF), bone morphogenetic proteins (BMPs) and IGFs, which either directly stimulate the osteoclast maturation or indirectly promote osteoclast differentiation through stimulating the osteoblasts to produce IL-6 and RANKL that stimulate osteoblast and osteoclast proliferation and maturation, leading to a net increase in osteoclast-mediated bone destruction or disorganized new bone formation. The reabsorption of bone matrix leads to the release of immobilized growth factors that stimulate tumor growth [TGF-β and insulin-like growth factor-1 (IGF1)].

The mutual stimulation between tumor and bone represents the rate-limiting step for tumor growth and contributes to chemotherapy resistance at the metastatic site. In osteoblastic lesions from prostate or hormone-dependent breast cancers, WNT signalling originating from tumor cells is essential to direct osteoblast differentiation by activating Runt-related transcription factor 2 (RUNX2) (2-3). Metastatic relapse can arise years after primary tumor removal since metastatic cells can remain dormant and resistant to conventional therapies (cancer stem cells) because the bone contains a unique hypoxic microenvironment replete with anti-apoptotic and survival signals, important for quiescent HSCs. A recent study with breast cancer models indicated that dormant cancer cells preferentially reside in the bone vascular niche, where the tumor cells keep quiescent through their adhesion to endothelial-derived thrombospondin-1 (6).

Exploration of the bone metastatic niche at structural, cellular and molecular levels is extremely useful to design novel therapies that treat incurable bone complications associated with cancer progression. These therapies will need to specifically target the mechanisms underlying tumor dormancy, metastatic relapse and therapeutic resistance. A better understanding of the bone metastatic niche will also be instructional in order to develop early interventions before the formation of macrometastasis as well as specific treatments for different stages of the bone metastasis.

Fracture risk estimation

Harrington and Mirels (7, 8) published the first studies on evaluation and diagnosis of impending fractures in the 80's. Harrington established the criteria to estimate the risk of an imminent fracture

while Mirels developed the first scoring system for decision-making about preventive surgery. However, over the years these criteria and scoring systems have been criticized for the lack of an external statistical validation and poor specificity in fracture risk estimation, often leading to an overstatement and therefore to an inadequate risk benefit assessment of surgical treatment.

In a recent paper, our study group have analysed the strengths and weaknesses of current methods for the diagnosis and indications to treatment of impending fractures (9) (Fig.1).

The use of software based CT images and more sophisticated models such as the finite elements analysis has greatly increased the accuracy of the fracture risk assessment. Tatar et al. (10) conducted a retrospective study using a virtual simulator based on CT images for the assessment of risk factors for impending fractures after radiation therapy in 47 patients treated for bone metastases. The parameters analysed were type of metastases (defined or shaded margins), appearance of metastases (normal, osteolytic, osteoblastic, mixed, and infiltrating), dimensional parameters (longitudinal, transverse axis, circumference), cortical thickness and extension of craniocaudal lysis. The results showed increased sensitivity and specificity for the thresholds of 45 mm for craniocaudal extension and 30% for circumferential involvement. However, the multivariate analysis revealed that only a circumferential involvement greater 30% was predictive of fracture.

Nowadays, the use of more sophisticated analytical models such as finite element has offered the best results in predicting fracture risk. Using a finite element model based on CT images Goodheart et al. (11) reproduced the forces acting on a metastatic bone during daily activities (axial load, stairs climbing and walking) and compared the results with the Mirels score. The results showed a superior predictive power of finite elements analysis, especially for loads acting during walking.

The main limitations of these sophisticated tools are the difficulties in their clinical application and the poor diffusion among the interdisciplinary figures involved in the management of these complex patients.

Not all osteolytic or pathologic lesions deserve a surgical treatment. The treatment of impeding fracture aims to prevent major complications for patients; it is therefore paramount to evaluate risks connected to the surgical treatment that must be significantly lower than expected benefits.

It is very difficult to assess the impact that surgery alone has on survival in this patient population. Few studies analysed the risk connected to the surgical treatment of bilateral pathologic fractures.

Moon et al. (12) evaluated the risk of death from pulmonary impairment and comorbidity in 16 patients treated with intramedullary nailing (with and without reaming) for bilateral pathologic fractures or impending fractures. Furthermore, they separately evaluated the group of patients treated with bilateral intramedullary nailing for impeding fractures to assess whether their risk was greater. This hypothesis derived from a previous study conducted by Kerr et al. (13) which showed an increase number of intraoperative deaths in patients treated for impending fractures (two deaths out of three patients), and therefore recommended a deferred nailing. However, the only survivor of these three patients did not receive a deferred nailing. The small number of patients treated for impending fractures led to an overstatement of the mortality rate in this subgroup of patients. The results of the study by Moon et al. (12) reported a death rate similar to the one of patients treated with single nailing, which ranges from 0% to 17%. Considering only patients who died intraoperatively for pulmonary embolism, mortality rate was 6% (one patient); this was similar to the rates reported for patients undergoing nailing for single impending or pathologic fracture, which ranges from 5% to 10%.

Economic impact of pathologic fractures

Hechmati G. et al. (14) conducted a multicentre prospective observational study involving Italy, Germany, Spain and the UK, to assess the costs weighting on social and health resources due to the SREs in patients with bone metastases from solid tumors and multiple myeloma. These costs were grouped into three classes: radiotherapy and interventional procedures, time of hospitalization, outpatient visits. The 66% of the total was related to radiation treatments and only 7% and 10% respectively to surgery for spinal cord compression

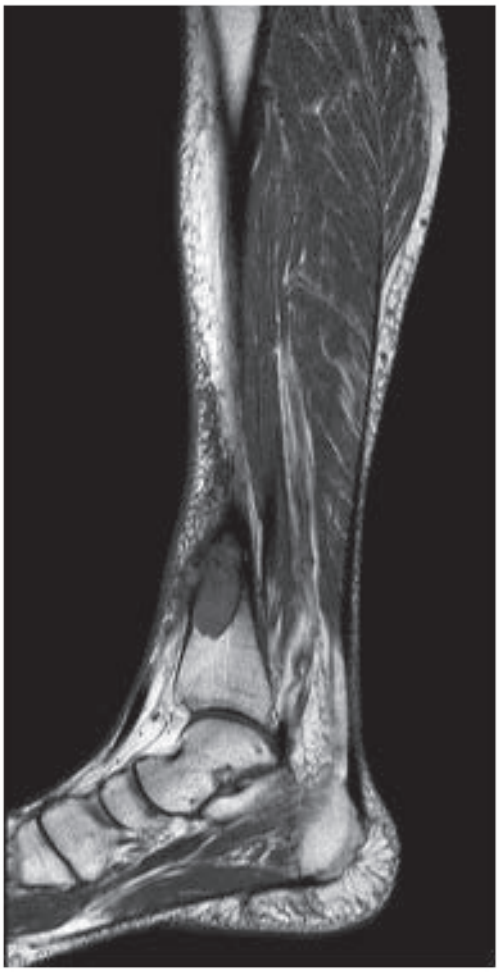


Fig. 1. *T1 weighted sagittal MRI of the distal tibia showing an impending fracture by a metastatic lesion of a thyroid carcinoma.*

and surgical procedures on the bone. The two latter however, involved a prolonged hospitalization time that represented the greatest cost in the management of these patients.

The authors therefore concluded that the effort to prevent or delay the occurrence of complications requiring prolonged hospitalization and long-term care is paramount in reducing social costs and healthcare resources consumption.

The concept of "impending fracture" and preventive treatment of fractures seems well aligned to this recommendations. However, it is fundamental to consider and include a careful evaluation of complications and risks relating to surgical procedures.

The accuracy of this evaluation, that reflects the decision criteria for the treatment of "impending fracture", and the choice of the best surgical treatment to minimise short and long-term risks for the patients are still unclear and often based on individual opinions.

Pathologic fractures and survival

The intervention of the orthopaedic surgeon on bone metastases always aims to improve the quality of life of patients (i.e. analgesic or palliative care). More recently, evidence suggests that new treatment strategies may also improve the survival.

Literature lacks consistent reports correlating the pathologic fractures to survival. No study correlates the treatment of "impending fracture" with increased survival. However, many studies report pathologic fractures as a negative prognostic factor for survival. Furthermore, there are indirect evidences that the treatment of "impending fractures" can have a positive correlation with survival. In a famous study by the Scandinavian Sarcoma Group, Hansen et al. (15) showed that a haemoglobin level lower than 7 g/dl is a negative prognostic factor for survival. The event of a pathologic fracture (in particular of large segments like the femur) can cause significant blood loss leading to haemoglobin level remarkably lower than 7 g/dl. The multivariate analysis showed that the presence of a complete pathologic fracture was a negative prognostic factor.

The blood loss consequent to a pathologic fracture implies the need for allogeneic blood transfusions. Janssen et al. (16) studied the correlation between the need of blood transfusions and survival in patients with pathologic fractures of the long bones from carcinoma metastases. Their retrospective study included 789 patients treated surgically in two centres. The results did not show a reduction in survival related to perioperative transfusions, however, the authors hypothesized that these results could affected by a numerical insufficiency of the sample. However, they observed a dose-dependent effect of transfusions, with patients who received more transfusions showing lower survival than those receiving less. The risk of death increased by 7% per unit of transfused blood.

Sugiura et al. (17) reported the results of a study

conducted on 1157 patients including 118 with bone metastases from lung cancer. In 70% of these patients survival was lower that one year, but in 6% of the cases it reached two years. Among the prognostic factors related to the bone the presence of solitary metastases, the absence of appendicular metastases and the absence of pathologic fractures positively correlated with survival.

Among the recent studies assessing survival, the one by Forsberg et al. (18) better discriminates the correlation between survival time and pathologic fractures. Through a Bayesian analysis of variables the study took into account two survival models with 3 and 12 months set as the end-points to opt for a lower-invasive treatment (intramedullary nailing or other simple osteosynthesis for patients with 3-12 months of life expectancy), or high invasivity (resection and prosthetic implant for patients with more than 12 months of life expectancy). The results showed that the variable "presence of pathologic fracture" was related in first degree in the three months model and in second degree in the 12 months model. This suggests that the presence of a pathologic fracture affects more significantly the survival of patients with worse prognosis (<12 months of life expectancy) and has a lower influence on those with better prognosis (>12 months of life expectancy). We have recently published the external validation of this method comparing three international patient population (19).

CONCLUSIONS

Pathologic fractures are dramatic events with a serious impact on the quality of life and survival of patients with bone metastases. Furthermore, pathologic fractures have relevant economic implications in the management of health resources. Given the predictability of these events, high accuracy in staging, diagnosis and treatment of impending fracture is recommended. Patients selection and choice of treatment considering expectancy of life, risks and expected benefits are both paramount. Referral centres should be in charge of diagnosis and treatment of the primary tumor; the treatment of bone metastases and pathologic fractures should not be delegated to other centres. For this reason, some European guidelines like those from the British Orthopaedic Association (www.boa.ac.uk) recommend to include an expert in metastatic bone disease in trauma teams.

REFERENCES

1. Piccioli A, et al. Documento SIOT sul trattamento delle metastasi ossee, GIOT vol. XXXVIII, Fasc. 5, ott 2012.
2. Ren G, Esposito M, Kang Y. Bone metastasis and the metastatic niche. J Mol Med 2015; epub ahead of print.
3. Esposito M, Kang Y. Targeting tumor-stromal interactions in bone metastasis. Pharmacol Ther 2014; 141:222-33.
4. Paget S. The distribution of secondary growths in cancer of the breast. Cancer Metastasis Rev 1989; 8:98-101.
5. Ell B, Mercatali L, Ibrahim T, Campbell N, Schwarzenbach H, Pantel K, Amadori D, Kang Y. Tumor-induced osteoclast miRNA changes as regulators and biomarkers of osteolytic bone metastasis. Cancer Cell 2013; 24:542-56.
6. Ghajar CM, Peinado H, Mori H, et al. The perivascular niche regulates breast tumour dormancy. Nat Cell Biol 2013;15:807-17.
7. Harrington KD. Impending pathologic fractures from metastatic malignancy: evaluation and management. Instr Course Lect. 1986;35:357-81.
8. Mirels H. Metastatic disease in long bones: A proposed scoring system for diagnosing impending pathologic fractures. Clin Orthop Relat Res 2003; 415(S):4-13.
9. Piccioli A, Spinelli MS, Maccauro G. Impending fracture: A difficult diagnosis. Injury 2014; 45(6):138-41.
10. Tatar Z, Soubrier M, Dillies AF, Verrelle P, Boisgard S, Lapeyre M. Assessment of the risk factors for impending fractures following radiotherapy for long bone metastases using CT scan-based virtual simulation: a retrospective study. Radiat Oncol 2014; 9:227.
11. Goodheart JR, Cleary RJ, Damron TA, Mann KA. Simulating activities of daily living with finite element analysis improves fracture prediction for patients with metastatic femoral lesions.J Orthop Res

2015: 8:1226-34
12. Moon B, Lin P, Satcher R, Lewis V. Simultaneous nailing of skeletal metastases: is the mortality really that high? Clin Orthop Relat Res 2011; 469:2367-70.
13. Kerr PS, Jackson M, Atkins RM. Cardiac arrest during intramedullary nailing for femoral metastases. J Bone Joint Surg Br 1993; 75(6):972-3.
14. Hechmati G, Cure S, Gouépo A. Cost of skeletal-related events in European patients with solid tumours and bone metastases: data from a prospective multinational observational study. J Med Econ 2013; 16(5):691-700.
15. Hansen BH, Keller J, Laitinen M, ET AL. The Scandinavian Sarcoma Group Skeletal Metastasis Register. Survival after surgery for bone metastases in the pelvis and extremities. Acta Orthop Scand Suppl 2004; 75(311):11-5.
16. Janssen SJ, Braun Y, Ready JE, Raskin KA, Ferrone ML, Hornicek FJ, Schwab JH. Are Allogeneic Blood Transfusions Associated With Decreased Survival After Surgery for Long-bone Metastatic Fractures? Clin Orthop Relat Res 2015; 473(7):2343-51.
17. Sugiura H, Yamada K, Sugiura T, Hida T, Mitsudomi T. Predictors of survival in patients with bone metastasis of lung cancer. Clin Orthop Relat Res 2008; 466(3):729-36.
18. Forsberg JA, Eberhardt J, Boland PJ, Wedin R, Healey JH. Estimating survival in patients with operable skeletal metastases: an application of a bayesian belief network. PLoS One 2011; 6(5):e19956.
19. Piccioli A, Spinelli MS, Forsberg JA, et al. How do we estimate survival? External validation of a tool for survival estimation in patients with metastatic bone disease-decision analysis and comparison of three international patient populations. BMC Cancer 2015; 15:424.

ASSESSING BONE MINERAL DENSITY IN ITALIAN ADULT CYSTIC FIBROSIS PATIENTS: A CROSS SECTIONAL STUDY

G. SOLARINO[1], G.VICENTI[1], A. SPINARELLI[1], A. ABATE[2], M. CARROZZO[1], V. CAGNETTA[3] and B. MORETTI[1]

[1]Department of Neuroscience and Organs of Sense, Orthopedics Section, Faculty of Medicine and Surgery, University of Bari, Bari, Italy; [2]Department of Orthopedics and Traumatology, Monsignor Raffaele Dimiccoli Hospital, Barletta, Italy; [3]Department of Neurology, Neurorehabilitation and Spinal Unit, IRCCS Maugeri Foundation, Cassano Murge, Italy

The purpose of this study was to assess bone mineral density in a cystic fibrosis (CF) outpatient clinic population and to investigate the relationship between BMD and forced expiratory volume in one second (FEV1), DEXA T-scores and 25-hidroxivitamin D (25-OHD) serum levels. We examined a consecutive series of 44 CF patients. Bone mass density was measured by dual-photon X-ray absorptiometry of lumbar spine and femur (total and neck) and lung function was performed in all patients. Medication data were obtained from medical records. A correlation analysis was performed to determine the relationship between BMD and forced expiratory volume in one second (FEV1), DEXA T-scores and 25-hidroxivitamin D (25-OHD) serum levels. In the results, age showed a significant inverse correlation indicating that as the age increases, bone density decreases and we concluded that most CF patients have low BMD and that there is a positive correlation with lung function and an inverse correlation with age.

Cystic fibrosis (CF) pathogenesis has been better investigated and understood during the last decades; increased life expectancy of patients favoured development of associated complications such as diabetes mellitus, pancreatic and liver disease, low bone mineral density and risk of fragility fractures (1, 2). Bone fractures can cause pain, decrease respiratory status and are a contraindication for lung transplantation in these patients (3, 4).

Cross-sectional studies in adults (age range from 16-60 years) with CF demonstrated that 6 to 68% had Z-scores ≤-2.0 (5-11) with an increased prevalence of osteopenia and osteoporosis in CF patients compared with the general population (12).

Bone disease in CF patients probably appears or worsens around puberty and continues deteriorating during adulthood with a variety of risk factors (13).

The etiology of osteoporosis in CF remains unknown, although factors such as corticosteroid use, vitamin D malabsorption, hypogonadism, inflammation, malnutrition and physical inactivity are thought to be involved (14-16). Puberty is a critical period for bone mineralization and requires special care so that optimum peak bone mass can be obtained. Thus, BMD gains are decreased even in young children with mild CF, suggesting the role of other pathophysiological mechanisms such as the negative impact of cystic fibrosis transmembrane conductance regulator (CFTR) protein dysfunction on bone formation (17).

Therefore effective strategic measures are necessary to optimize bone health, such as BMD

Key words: cystic fibrosis, bone mineral density, T-Score, 25 OH-Vitamin D, FEV_1

Mailing address: Giovanni Vicenti, MD,
Department of Neuroscience and Organs of Sense,
Orthopedics Section, Faculty of Medicine and Surgery,
University of Bari, Policlinico,
Piazza Giulio Cesare 11, 70124 Bari, (BA), Italy
Tel.: +393480128360
e-mail: dott.gvicenti@gmail.com

0393-974X (2015)

monitoring by bone densitometry (DXA) and preventive care from infancy through adolescence (18).

The purpose of this study was to assess bone mineral density in a cystic fibrosis outpatient clinic population and to investigate the relationship between BMD and forced expiratory volume in one second (FEV1), T-Scores and 25-hidroxivitamin D (25-OHD) serum levels.

MATERIAL AND METHODS

In this cross-sectional study, the samples consisted of patients with clinical diagnosis of CF genetically confirmed and followed at the CF clinic of the Policlinico of Bari.

Patients were aged between 20 and 44 years, had a clinically stable disease and cognitive capacity to perform all procedures. Patients who failed to perform the lung function test or who were receiving systemic corticosteroids for more than 15 consecutive days were excluded.

The Research Ethics Committee of Policlinico di Bari initiated the research protocol only after approval and an informed consent form was read and signed by patients and/or guardians.

Firstly, data were collected regarding patient identification (name, medical record number, age and gender), weight, and height. Selected patients underwent pulmonary function test (spirometry), DXA examination and Serum 25(OH) vitamin D (25-OHD) levels were measured by chemiluminiscence as routine outpatient care. Additional data were obtained from the outpatient clinic files which are regularly updated.

Pulmonary function test

Assessment of pulmonary function was performed by spirometry using a "FUKUDA MOD. ST 250" spirometer. The evaluated spirometric parameters included forced vital capacity (FVC), forced expiratory volume in one second (FEV1), Tiffeneau index (FEV1/FVC) and forced expiratory flow 25% and 75% of FVC (FEF 25%-75%).

Calibration was performed before each testing session according to the manufacturer's instructions. In order to perform the spirometric test, patients should not have had exacerbation of respiratory symptoms nor have used short-acting bronchodilator 4 h before the test or long-acting bronchodilator 12 h before the test. A resting period of 5 to 10 min was required before each test.

After instructions and training, patients were asked to start the exam. The spirometric test was described in detail to each patient, with emphasis on maximum inspiration followed by a maximal fast expiration, following the criteria established by the American Thoracic Society (ATS) (19). All spirometric tests were performed individually, in upright position, with the use of a nose clip.

Bone densitometry

DXA for the assessment of bone mineral content was performed by dual-energy X-ray absorptiometry in a Hologic Discovery Wi DXA scanner. All tests were performed with the same unit at our Orthopaedic Department. Bone density was measured at the lumbar spine (LS) (L1-L4) as well as the femoral neck (FN) and was expressed as the number of grams of bone mineral per square centimetre of bone. The data were also expressed as a Z-score and a T-score. A T-score is the number of standard deviations (SDs) below the mean score for young adult control subjects, while the Z-scores reflects the number of SDs below the mean of age-matched control subjects.

Osteopaenia is defined according to the World Health Organization as being present if the T-score is between -1.0 and -2.5 and osteoporosis is defined as a T-score that is <-2.5 (20). Z-score is an important value to consider in patients affected by CF. When results are -2 or lower, it may suggest that something other than aging is causing abnormal bone loss.

Serum

Blood was drawn for biochemistry measurements 25-OHD which is the most accurate measure of the amount of vitamin D in the body.

Statistical analysis

Patients' details was collected in a chart and then summarized in a pro database file and analysed. In order to have continuous data, means, standard deviations (SD) and medians were calculated. Pearson correlation coefficients were checked to determine the relationship between BMD and forced expiratory volume in one sec (FEV1), T-Scores and 25-hidroxivitamin D (25-OHD) serum levels. Student's *t*-test was used for statistical analysis. For all tests a p-value <0.005 was considered statistically significant.

RESULTS

Forty-four patients with CF were included, with a mean age of 30.4±6.0 years (range 20-44); 24 (54.55%) were males. The sample characterization are shown in Table I. The Z (-0.1±1.8) and T score (-1.141±1.8) distributions show that average values

Table I. *Patients Characteristics.*

Variables	
Age (Years)	30.4±6.0
Gender	
Male Female	24 (54.55%) 20 (45.45%)
BMI	23.12±2.6
FEV1(%)	67.98±10.66
BMD (g/cm2)	0.791±0.273
Z-score	-0.1±1.8
T-score	-1.141±1.8
25-OH-Vit.D	12.75±4.14

are below normal range (Fig. 1, 2).

Lung function assessment showed that 86% of patients (38) had FEV1 below 80% and within this group, four patients had a FEV1 under 50%. The mean serum 25-OHD concentration (12.57ng/ml) was at the low end of the normal range (10 to 60 ng/ml) (21); 8 of 44 patients (18%) had very low (<10 ng/ml) levels.

Age showed a significant inverse correlation (r=−0.84, p=0.004), indicating that as the age increased bone density decreased (Fig. 3, 4).

When analysed individually FEV1 was positively associated with BMD at the LS and the FN as demonstrated by the Correlation Coefficient (r=0.881; p=0.0041) (Fig. 5). When correlating

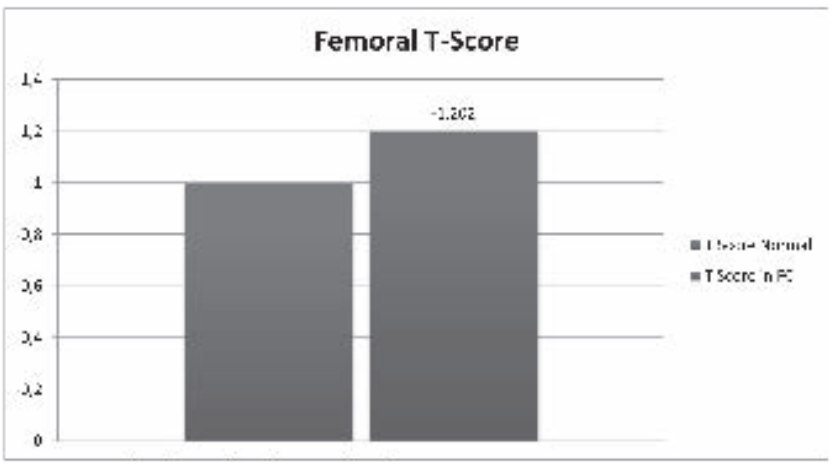


Fig. 1. *Femoral T-Score of population of study.*

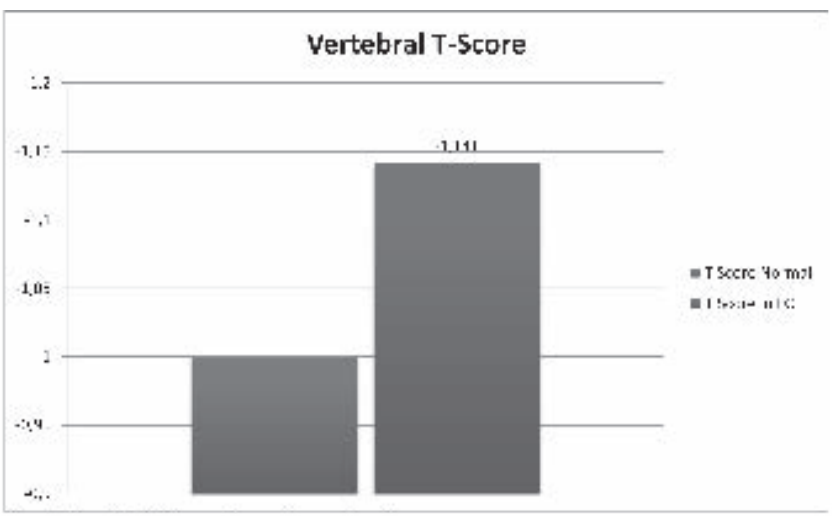


Fig. 2. *Vertebral T-Score of population of study.*

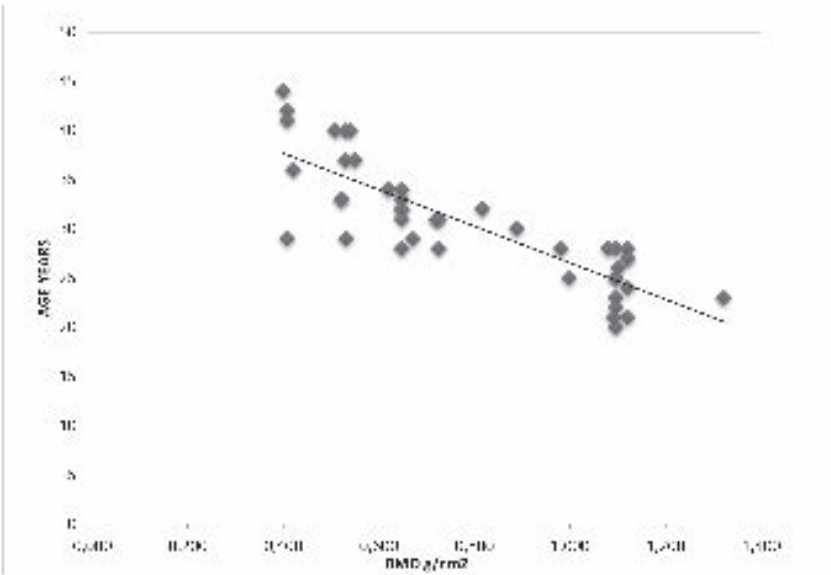


Fig. 3. *Correlation between age and BMD (**r**=−0.84; **p**=0.004).*

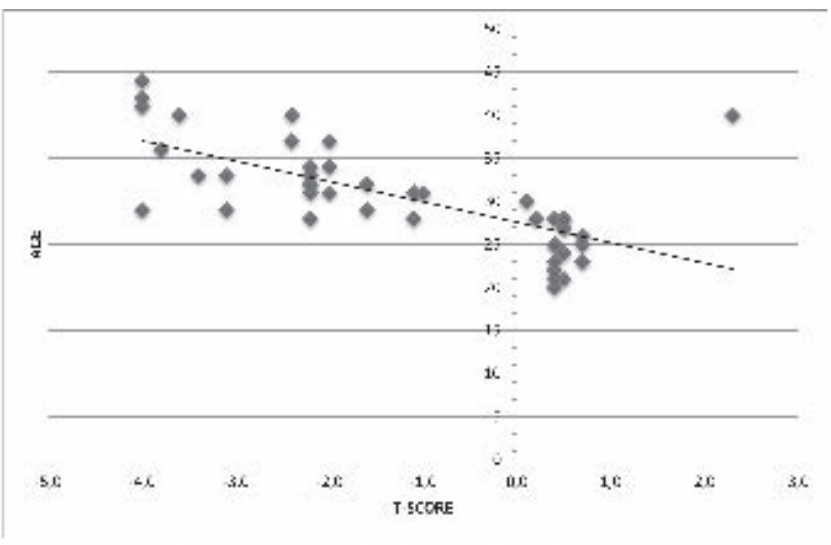


Fig. 4. *Correlation between age and t-score (**r**=-0.68; **p**=0.00472).*

FEV1 data and t-score values obtained in the DXA test, a moderate correlation coefficient was observed (r= 0.643, p=0.003) (Fig. 6). No positive correlation was found between 25-OHD and Femoral T-score (Fig.7). In patients with mild mutation, the values were similar to those with severe mutation. However, it cannot be excluded that this result was due to the small size of the sample. Finally, we found normal values of BMD and T-Score in 5 patients who have constantly practiced sport during the pubertal period.

DISCUSSION

In the present study, CF outpatients had bone

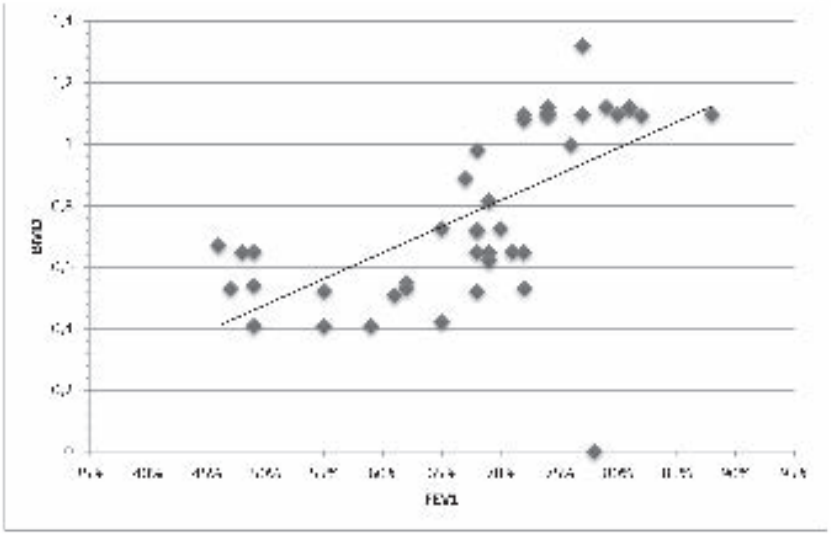


Fig. 5. *Correlation between BMD and FEV1 (**r**=0.881; **p**=0.0041).*

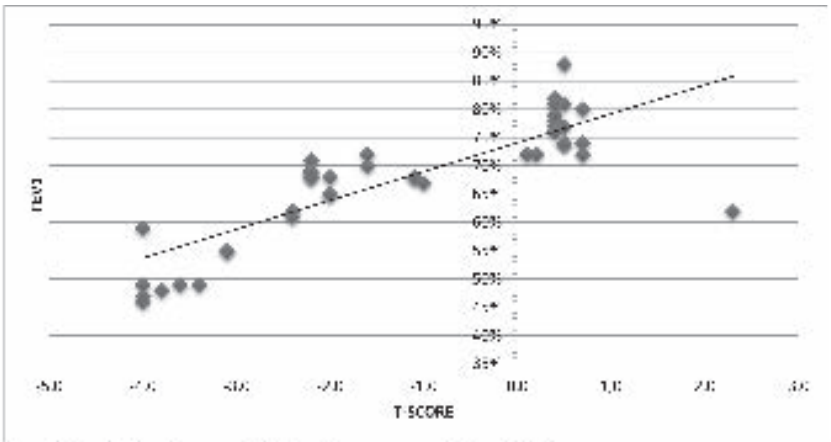


Fig. 6. *Correlation between FEV1 and T-score (**r**=0.643; **p**=0.003).*

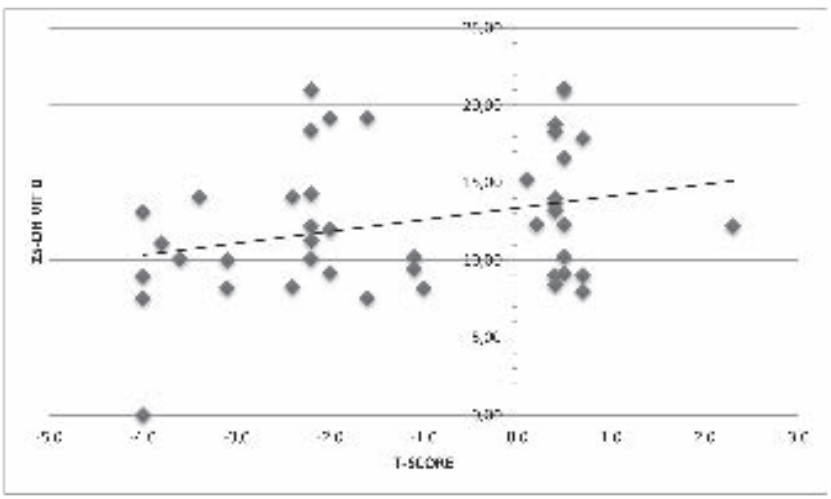


Fig. 7. *Correlation between 25-OH and t-score (**r**=0.15; **p**=0.005*

mass below the expected range when considering both t and z score at lumbar spine and femur. In addition, they presented a positive correlation with lung function and an inverse correlation with chronological age.

Previous studies reported normal BMD values and attributed these findings mainly to the preservation of nutritional status and low pulmonary impairment. However, several studies (11, 21, 22) reported BMD deficiency in children and linked this decrease to nutritional impairment, number of hospitalizations, worse lung function, gonadal dysfunction and treatment with steroids.

Moreover, these contrasting results appear to be related to the lack of uniformity in study population. However decreased bone mass in adults appears more present and to have increased due to rise in patient survival rate (12, 23) .

Lung function as evaluated throughout FEV1 generally showed a positive correlation with BMD values expressed with both t and z scores; this explains why worse the lung function, the lower the BMD indices.

These results are in agreement with several authors who demonstrated FEV1 as a robust marker of lung disease severity, establishing a direct association with bone mass at all ages (18, 23).

Pulmonary damage due to tissue inflammation post colonisation leads to increased in cytokines, which precedes lung function impairment. These cytokines stimulate bone resorption which may be a contributing factor in the association between disease severity and BMD (18, 24).

Of the nutritional factors associated with bone mass, the most important are vitamin D, calcium ingestion, and BMI (4). Although 25-OHD was below 30ng/mL, no correlation was found between femoral T score and 25-OHD levels, according to previous studies (25).

This contributes to the issue concerning the pathogenesis of low BMD in these patients, which is still not completely understood. As mentioned, calcium and vitamin D malabsorption, malnutrition, low BMI, delayed puberty, hypogonadism and glucocorticoid intake contribute to low BMD. However, recent studies in bone cells of CF patients show the loss of CFTR activity that may result in an increased inflammation-driven bone resorption (through both the reduced OPG and increased PGE2 release). This might contribute to the low bone mineral density found in young children with CF, independent of their nutritional status and the severity of lung disease (26).

Donadio et al. (13) showed an inverse correlation with age at diagnosis, showing that the older the age at which the diagnosis is made, the lower the bone density values, which demonstrates the importance of neonatal screening and of early diagnosis that would enable the rapid inclusion of these patients in referral centers for disease treatment and monitoring. Regular exercise and physical activity are important for patients with cystic fibrosis (CF). Research demonstrated the benefits of aerobic, anaerobic and strength exercise training programs for health and quality of life in CF patients; this promotes mucus clearance, preventing lung recurrent infection and reducing chronic inflammation citokines production, which are associated with bone resorption. This also promotes better glicemic control in patients with pancreatic insufficiency and bone peak mass achieving.

The present study demonstrates that most CF patients have low BMD and that there is a positive correlation with lung function and an inverse correlation with age. The limitation of our study is the small sample size. Additionally, important variables such as age at diagnosis and comorbidities were not evaluated.

Longitudinal and cross sectional studies on larger sample size are necessary to obtain further information and to promote comprehension of factors that may influence the evolution of bone health to prevent and improve quality of life of patients with CF.

REFERENCES

1. Pedreira CC, Robert RGD, Dalton V, et al. Association of body composition and lung function in children with cystic fibrosis. Pediatr Pulmonol 2005; 39:276-80.
2. Douros K, Loukou I, Nicolaidou P, Tzonou A, Doudounakis S. Bone mass density and associated factors in cystic fibrosis patients of young age. J Paediatr Child Health 2008; 44:681-5.

3. Paccou J, Zeboulon N, Combescure C, Gossec L, Cortet B. The prevalence of osteoporosis, osteopenia, and fractures among adults with cystic fibrosis: A systematic literature review with meta-analysis. Calcif Tissue Int 2010; 86:1-7.
4. Spira A. Osteoporosis and lung transplantation: a prospective study. Chest 2000; 117(2):476-81.
5. Elkin SL, Fairney A, Burnett S, et al. Vertebral Deformities and Low Bone Mineral Density in Adults with Cystic Fibrosis: A Cross-sectional Study. Osteoporos Int 2001; 12:366-72.
6. Robertson J, Macdonald K. Prevalence of bone loss in a population with cystic fibrosis. Br J Nurs 2010; 19:636-9.
7. Dodd JD, Barry SC, Barry RBM, Cawood TJ, McKenna MJ, Gallagher CG. Bone Mineral Density in Cystic Fibrosis: Benefit of Exercise Capacity. J Clin Densitom 2008; 11:537-42.
8. Street ME, Spaggiari C, Ziveri MA, et al. Analysis of Bone Mineral Density and Turnover in Patients with Cystic Fibrosis: Associations between the IGF System and Inflammatory Cytokines. Horm Res 2006; 66:162-8.
9. King SJ. Reduced bone density in cystic fibrosis: F508 mutation is an independent risk factor. Eur Respir J 2005; 25:54-61.
10. Frangolias DD, Paré PD, Kendler DL, et al. Role of exercise and nutrition status on bone mineral density in cystic fibrosis. J Cyst Fibros 2003; 2:163-70.
11. Bhudhikanok GS, Lim J, Marcus R, Harkins A, Moss RB, Bachrach LK. Correlates of osteopenia in patients with cystic fibrosis. Pediatrics 1996; 97:103-11.
12. Haworth CS, Selby PL, Webb AK, Dodd ME, Musson H, McL Niven R, et al. Low bone mineral density in adults with cystic fibrosis. Thorax 1999; 54:961-7.
13. Donadio MVF, De Souza GC, Tiecher G, , et al. Bone mineral density, pulmonary function, chronological age, and age at diagnosis in children and adolescents with cystic fibrosis. J Pediatr (Rio J) 2013; 89:151-7.
14. Haworth CS, Selby PL, Webb AK, Adams JE. Osteoporosis in adults with cystic fibrosis. J R Soc Med 1998; 91 Suppl 3:14-8.
15. Ott SM, Aitken ML. Osteoporosis in patients with cystic fibrosis. Clin Chest Med 1998;19:555-67.
16. Lambert JP. Osteoporosis: a new challenge in cystic fibrosis. Pharmacotherapy 2000; 20:34-51.
17. Javier R-M, Jacquot J. Bone disease in cystic fibrosis: What's new? Jt Bone Spine 2011; 78:445-50.
18. Sermet-Gaudelus I, Castanet M, Retsch-Bogart G, Aris RM. Update on cystic fibrosis-related bone disease: a special focus on children. Paediatr Respir Rev 2009; 10:134-42.
19. Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, et al. Standardisation of spirometry. Eur Respir J 2005; 26:319-38.
20. Looker AC, Orwoll ES, Johnston CC, et al. Prevalence of low femoral bone density in older U.S. adults from NHANES III. J Bone Miner Res 1997; 12:1761-8.
21. Ujhelyi R, Treszl A, Vásárhelyi B, Holics K, Tóth M, Arató A, et al. Bone mineral density and bone acquisition in children and young adults with cystic fibrosis: a follow-up study. J Pediatr Gastroenterol Nutr 2004; 38:401-6.
22. Gronowitz E, Garemo M, Lindblad A, Mellström D, Strandvik B. Decreased bone mineral density in normal-growing patients with cystic fibrosis. Acta Paediatr 2003; 92:688-93.
23. Legroux-Gérot I, Leroy S, Prudhomme C, et al. Bone loss in adults with cystic fibrosis: Prevalence, associated factors, and usefulness of biological markers. Jt Bone Spine 2012; 79:73-7.
24. Conway SP. Impact of lung inflammation on bone metabolism in adolescents with cystic fibrosis. Paediatr Respir Rev 2001; 2:324-31.
25. Vanacor R, Raimundo F V, Marcondes NA, Corte BP, Ascoli AM, Azambuja AZ de, et al. Prevalence of low bone mineral density in adolescents and adults with cystic fibrosis. Rev Assoc Med Bras 2014; 60:53-8.
26. Le Heron L, Guillaume C, Velard F, Braux J, Touqui L, Moriceau S, et al. Cystic fibrosis transmembrane conductance regulator (CFTR) regulates the production of osteoprotegerin (OPG) and prostaglandin (PG) E2 in human bone. J Cyst Fibros 2010; 9:69-72.

VITAMIN D DEFICIENCY IN ADULTS: SEARCHING FOR THE PROPER LOADING DOSE

G. VICENTI[1], V. PESCE[1], A. ABATE[2], M. CARROZZO[1], V. CAGNETTA[3], F. RIFINO[1], G. SOLARINO[1] and B. MORETTI MD[1]

[1]Department of Neuroscience and Organs of Sense, Orthopedics Section, Faculty of Medicine and Surgery, University of Bari, Bari, Italy; [2]Department of Orthopedics and Traumatology, Monsignor Raffaele Dimiccoli Hospital, Barletta Italy; [3]Department of Neurology, Neurorehabilitation and Spinal Unit, IRCCS S. Maugeri Foundation, Cassano Murge, Italy

Vitamin D is the main hormone regulating calcium phosphate homeostasis and mineral bone metabolism. Vitamin D deficiency is indeed extremely frequent in musculoskeletal diseases. Recent studies have shown that the treatment of osteoporosis needs to have an optimal vitamin D and calcium supplementation for its efficacy(7). Actually no agreement exists on the estabilished dose of vitamin D to administer in deficency states. We conducted a prospective study to develop a practical cholecalciferol loading dose regimen that would enable rapid correction of vitamin D deficiency. Sixty post-menopausal age woman were enrolled secondary to a fragility fracture (hip, vertebral, wrist) and screened for 25-hydroxyvitamin D (25(OH)D), calcium, and PTH at baseline (T0), after one month (T1), two months (T2), three months (T3) and six months (T4). Secondary to initial blood values of vitamin D patients were divided into 2 groups; the first group (group A, n=30) included patients with 25(OH)D values between 10-30 ng/ml and the second group (group B, n=30) with values under 10 ng/ml. Each group was then divided in 3 subgroups secondary to the randomized administered dose of 25(OH)D. By this, patients can alternatively receive 25000 UI two times monthly, 100000 UI monhtly, 10000 UI (25 drops) weekly. The highest values of mean increase of 25(OH)D were observed in patients treated with 100000 UI. Patients treated with 10000 UI weekly did never achieve the target value. Additionally, as vitamin levels increased, pain intensity decreased. Vitamin D supplementation of 100000 UI monthly seems to be adequate to ensure that serum 25(OH)D values reach the threshold level; by this, it will confer the expected effects without risks of toxicity.

Vitamin D is the main hormone regulating calcium phosphate homeostasis and mineral bone metabolism. The discovery that a variety of tissues can express vitamin D receptor (VDR) has opened new ways of research related to vitamin D biological effects and molecular pathways (1-3). There is evidence that vitamin D is implicated in the regulation of both immune and cardiovascular systems, oncogenesis (4) and cognitive functions (5).

Vitamin D deficiency is indeed extremely frequent in musculoskeletal diseases. The clinical observation that patients with rickets and osteomalacia displayed proximal myopathy suggested a direct link between hypovitaminosis D and muscle function (6).

Recent evidence has confirmed that vitamin D may modulate muscle growth. Consequently

Key words: Bone health, musculoskeletal health, osteoporosis, Vitamin D, cholecalciferol, 25-hydroxycholecalciferol

Mailing address: Giovanni Vicenti, MD,
Department of Neuroscience and Organs of Sense,
Orthopedics Section, Faculty of Medicine and Surgery,
University of Bari, Policlinico,
Piazza Giulio Cesare 11, 70124 Bari, Italy
Tel.: +393480128360
e-mail: dott.gvicenti@gmail.com

0393-974X (2015)

the effect of vitamin D on skeletal muscles and its clinical implications, especially frailty and the risk of fall, are important to know. Vitamin D levels are based upon correspondence levels of parathyroid hormone (PTH). Low vitamin D levels starts a feedback mechanism which stimulates PTH to rise.

Recent studies have shown that the treatment of osteoporosis with bisphosphonates needs to have an optimal vitamin D and calcium supplementation for their efficacy (7).

The aim of this study was to develop a practical cholecalciferol loading dose regimen that would enable rapid correction of vitamin D deficiency. We evaluated the relationship beetween low circulating levels of 25(OH)D and chronological age, VAS score, secretion of parathyroid hormone (PTH).

MATERIAL AND METHODS

Partecipants

Sixty post-menopausal-age woman (mean age 53-90 years) who had been admitted to our Orthopedics Departement at the Policlinico Bari after a fragility fracture (hip, vertebral, wrist) between July 2009 and December 2011, were enrolled. During the hospitalization, they were tested for vitamin D deficiency. Patients with unstable thyroid disorders, hypoparathyroidism and primary hyperparathyroidism, evidence of malabsorption, and renal insufficiency (glomerular filtration rate<60 ml/min) and those who used medication interfering with vitamin D metabolism were excluded. All patients gave their informed consent.

For the analysis of serum 25(OH)D, creatinine, calcium, phosphate, albumin and PTH, blood samples were collected at baseline (T0), after one month (T1), two months (T2), three months (T3) and six months of treatment (T4).

Serum 25(OH)D concentrations were measured by RIA (DiaSorin, Stillwater, MN, USA). Serum intact PTH was measured by chemiluminescent enzyme-labeled immunometric assay (Immulite 2500, Siemens, Los Angeles, CA, USA).

According to initial blood values of vitamin D, patients were divided into 2 groups. The first group (group A, n=30) included patients with 25(OH)D values between 10-30 ng/ml and the second group (group B, n=30) with values under 10 ng/ml.

Each group was then divided in 3 subgroups following the randomized administered dose of vitamin D. Patients can alternatively receive 25000 UI two times monthly, 100000 UI monhtly, 10000 UI (25 drops) weekly.

The solubilized cholecalciferol is a watery mixture made of cholecalciferol concentrate in oil, citric acid monohydrate, star anise oil, potassium sorbates, polysorbatum 80 (polyoxyethylene sorbitan mono-oleate), sugar syrup and purified water. Cholecalciferol is a fat-soluble vitamin that is solubilized by polysorbatum 80, an interface-active substance that causes micelle formation. After oral administration, the solubilisate changes into a very fine emulsion with better bioavailability than oily formulations. All patients also received a daily calcium dose of 1000 mg. VAS evaluation was performed at T0 and when the 25(OH)D threshold values were rated.

Statistical analysis

Results are expressed as mean values±S.D. Data that did not follow a normal distribution were log-transformed. Responses to treatment within groups were analyzed by paired, two-sided *t*-tests. Between-group differences were tested by ANOVA and unpaired t-tests. To enable calculation procedures, 25(OH)D levels below the assay's detection limit were given a value of 2 ng/ml. Multivariate regression analysis was used to identify the variables that might predict the cholecalciferol loading dose. Variables included for this analysis were season, sex, age, height, body weight, body mass index (BMI), cholecalciferol dose in IU and in IU/kg body weight, and baseline serum 25(OH)D. To obtain a simple model that would be easy to use in daily practice, only those variables explaining more than 5% of the total variance were considered to be clinically relevant, and variables explaining <5% of the variance were removed. A P value <0.05 was considered to be statistically significant.

RESULTS

The mean age of group A patients was 64.27 years with a mean value of 19.08 ng/ml of 25(OH) D at t0. Group B patients had a mean age of 76.26 years with a mean value of 6.35 ng/ml of 25(OH) D. The mean value of 25(OH)D was of 13.02 ng/ml.

The laboratory results at baseline and at each time of the study, are summarized in Table I (insufficiency vit D group A) and Table II (deficiency vit D group B). In group A, 9 patients achieved threshold within 1 month; 8 patients had been treated with a dose of 100000 UI monthly; 1 patient with 25000 UI two times a month. All 30 patients of group A achieved threshold values within 3 months.

In group B, no patients achieved threshold rate within the first month. All patients treated with 100000 UI achieved the target values within 6

months. Patients treated with 10000 UI weekly never achieved the target value. Hypercalcemia, defined as a serum calcium level >2.55 nmol/l, was not observed.

The highest values of mean increase of 25(OH) D (18.04 ng/ml)were observed in Group A patients when treated with 100000 UI at one month. Even in the Group B patients, the highest values of increase (30 ng/ml) were achieved when treated with 100000 UI monthly at a mean of three months (Table III-IV).

When these values were compared, the mean rise in serum 25(OH)D in group B was greater than that in group A ($P<0.05$).

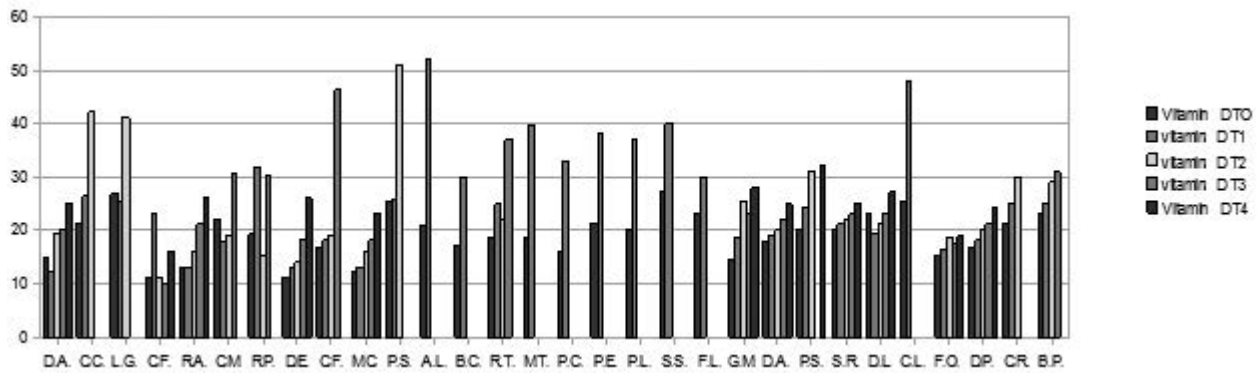
A statistically significant decrease in serum PTH was observed only at T4 of the study (5.5±5.3–4.0±2.8, $P<0.01$), and complete suppression of serum PTH did not occur.

Statistical analysis showed positive correlation between chronological age and 25(OH)D (Table V).

Lower levels of 25(OH)D at T0 were associated with higher pain intensity at VAS score. As vitamin levels increase, pain intensity decreases (Table VI a-c).

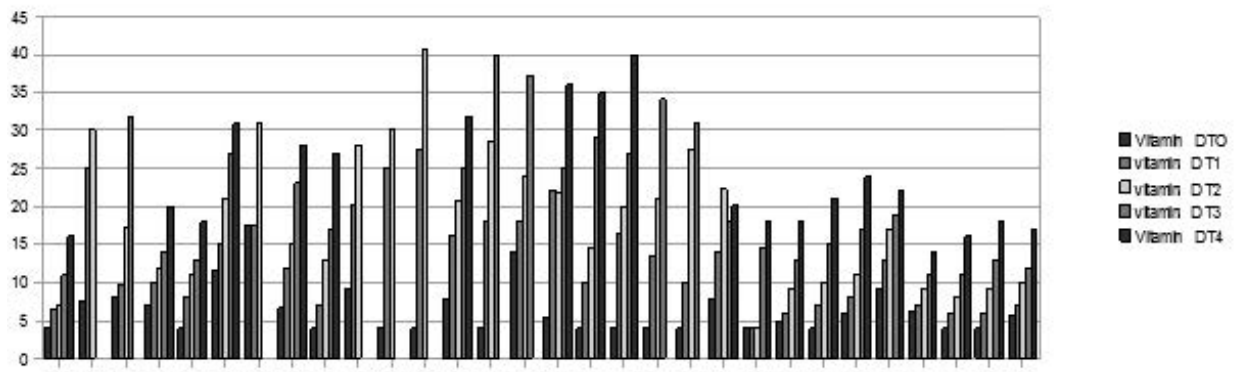
No statistical significance was observed when correlating vitamin D levels with both PTH and Calcium levels at T0. On the other hand PTH values showed a minimal correlation with Calcium levels at T0 (Table VII).

Table I. *Group A Vitamin D levels at each time of the study*



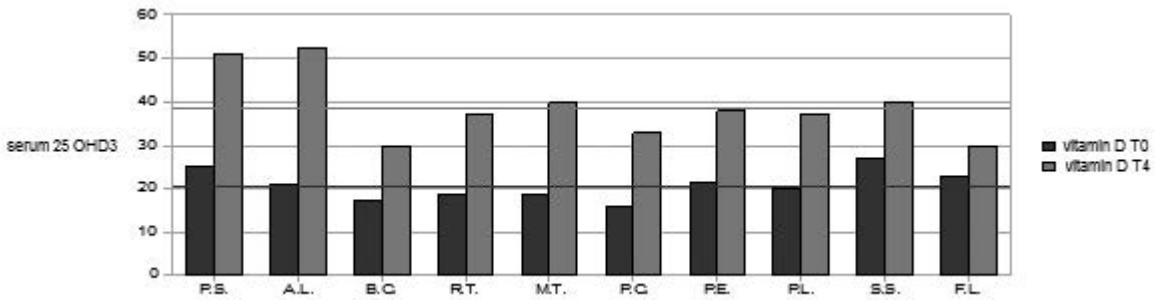
Increase in Vitamin D levels for each group A patients at different times since threshold was achieved.

Table II. *Group B Vitamin D levels at each time of the study*



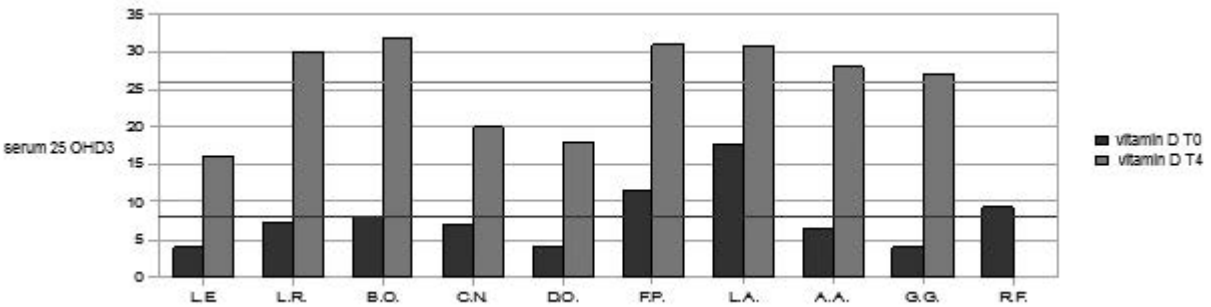
Increase in Vitamin D levels for each patient in group B at different times since threshold was achieved.

Table III. *Vitamin D levels at T0 and T4.*



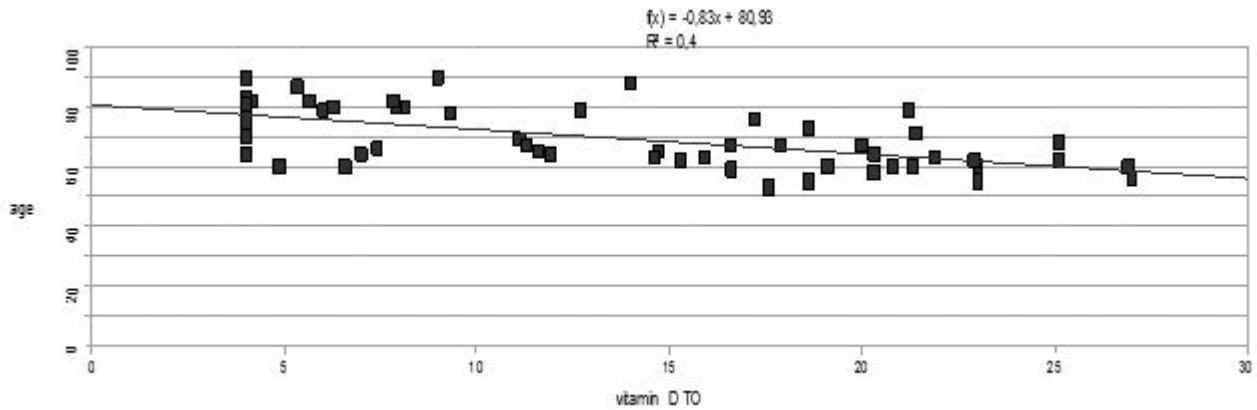
Group A patients receiving full doses of Vit D (100000/month) showed the highest values of increase.

Table IV. *Vitamin D increase in group B patients.*



Group B patients receiving full doses of Vitamin D (100000/month) showed the highest values of increase.

Table V. *age and vitamin D correlation at T0 R=0.4.*



Correlation was observed among chronological age and 25-OHD3at T0.

Table VI. *a)*: *T0 and VAS1 correlation p<0.0001.*

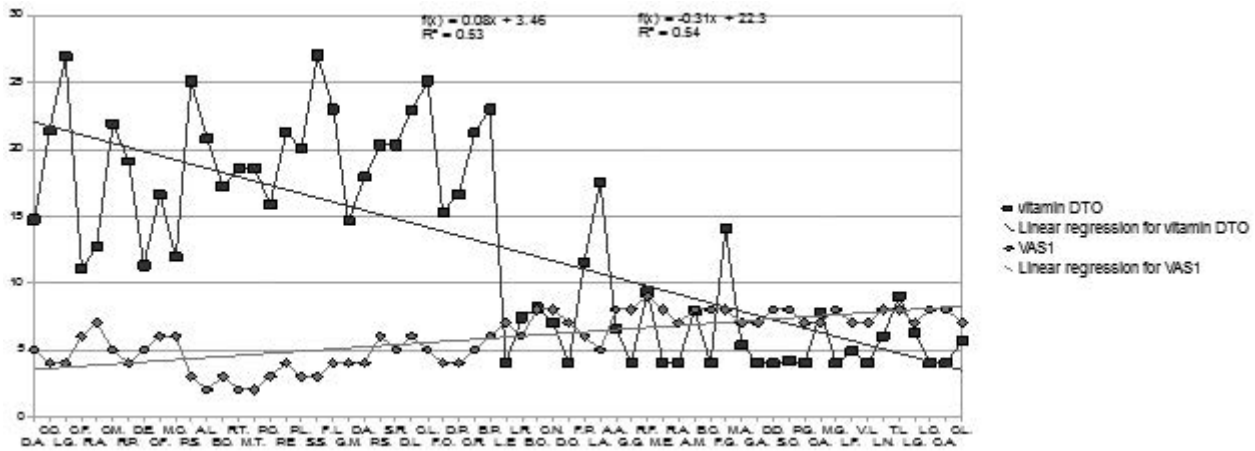


Table VI. *b)*: *Vitamin D T0 and VAS1 correlation R2=0.62*

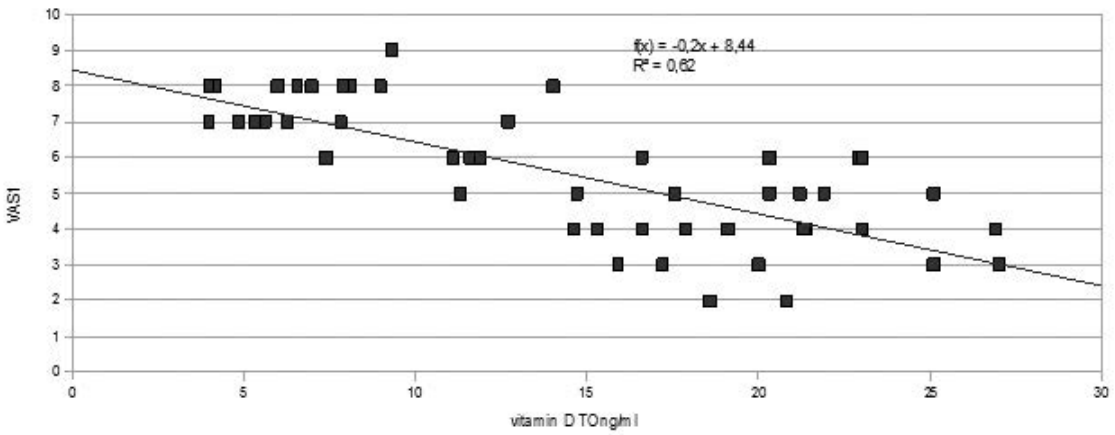


Table VI. *c)*: *Vit D at T4 and VAS2 correlation R2=0.38.*

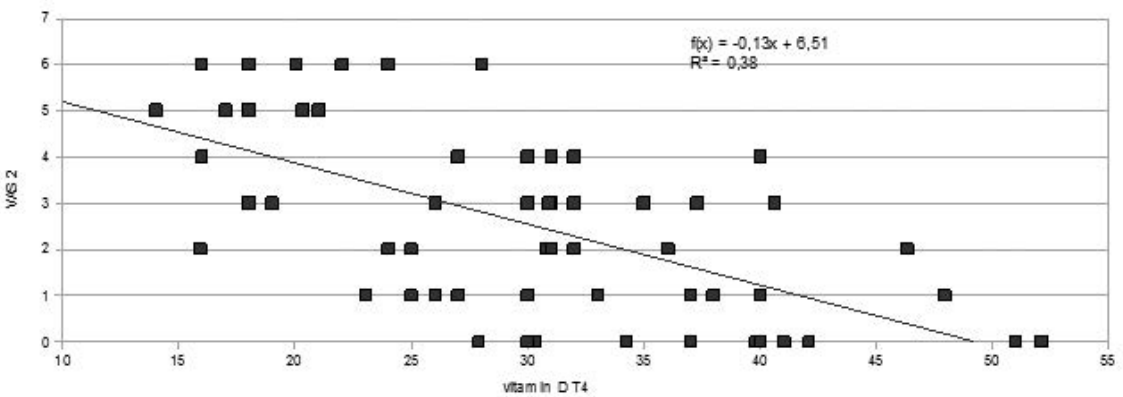
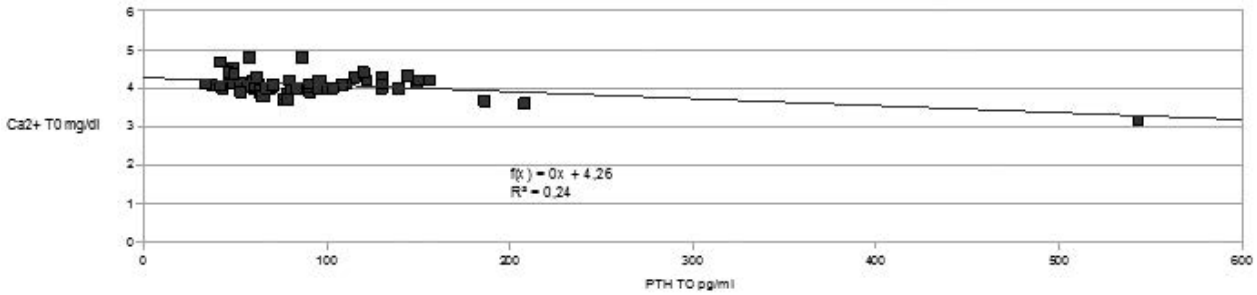


Table VII. *PTH and Calcium correlation at T0*



Calcium levels showed correlation with PTH levels at T0.

DISCUSSION

This study formulated a loading dose guideline for rapid correction of vitamin D deficiency using solubilized cholecalciferol. The calculation of the dose required to normalize serum 25(OH)D is based on the degree of vitamin D deficiency. It appears to be a safe procedure. In this study, no toxic effects were observed, hypercalcemia did not develop, vitamin D levels never reached the danger range and complete suppression of PTH levels was not observed.

The sampling was well distributed over all seasons including summer, which further supports the safety of the proposed regimen. The study included post-menopausal woman, lean and obese, with a variety of underlying diagnoses such as those commonly fracture of our orthopaedics department. Once the target level is reached, a maintenance dose will be required to sustain the serum 25(OH)D level around 30 ng/ml.

Until today, vitamin D supplementation studies were mainly limited to elderly people, particularly those who lived in nursing homes where the prevalence of vitamin D deficiency is 75% or higher. Some studies evaluated the efficacy of high doses at monthly or 3-month intervals, but most studies have used the officially recommended cholecalciferol dose of 600–800 IU/day, or equivalents (8-12).

Most studies used predefined fixed doses, irrespective of the degree of vitamin D deficiency and body weight and assessed the efficacy of the regimen after 4-6 months or longer. These studies were not designed to establish the requirements for a loading dose. A dose of 800 IU/day may help to improve vitamin D status in the long run, but it is not the optimal approach to achieve a rapid correction of severe vitamin D deficiency (12).

According to our calculations, a patient with a serum 25(OH)D level <10 ng/ml will need a total loading dose of 100 000 IU and the patients with vitamin D deficiency need a total dose of 100000 UI to normalize the 25(OH)D levels.

Discussions on vitamin D requirements and daily allowances are not new. Over the years, the recommended daily dose for adults has gradually increased from 200 to 800 IU and recently, doses of 1000 and even 2000 IU/day or higher have been proposed (13, 14).

Vitamin D supplementation must be sufficient to ensure that serum 25(OH)D values reach the threshold level, otherwise it will not confer the desired benefit. Studies investigating the anti-fracture efficacy of different dosing regimens of vitamin D have shown that 400 IU per day was not sufficient to have an effect on fracture rate (15). Oral doses of N700-800 IU taken daily or 100,000 IU taken quarterly both showed a positive anti-fracture effect, whereas an intramuscular dose of 300,000 IU annually showed inconsistent efficacy (15, 16). This suggests that supplementation is most effective in osteoporotic patients if given orally either daily or quarterly, and if given daily, should be in excess of 700-800 IU daily (17).

Vitamin D toxicity is often feared, but usually is unfounded. All published cases of vitamin D toxicity involved intakes of more than 40 000 IU/day

for a prolonged period of time, with serum 25(OH) D levels exceeding >100 ng/ml. To achieve these levels, daily doses of 15000 IU or more for many months are needed. The lowest cumulative dose of cholecalciferol leading to toxic 25(OH)D levels was 3600000 IU given in 3 weeks (13). The loading doses that we have proposed are 10-20 times less.

In the present study, the best way to treat vitamin D deficiency was to give cholecalciferol in doses of 10000 IU/months with calcium supplementation.

Current evidence suggests that the role of calcium and vitamin D play in fracture prevention is not attributable to calcium alone (18, 19) and a meta-analysis of data from 9 randomized clinical trials, including a total of 53.260 patients found that where the effects of supplementation with vitamin D alone were explored (in a total of 9038 patients), this was not sufficient to significantly reduce the risk of hip fracture in postmenopausal women (20). However the same study found that combined supplementation with vitamin D and calcium reduced the risk of hip fracture by 25% (95% CI: 4-42) and the risk of non-vertebral fracture by 23% (95% CI: 1-40) compared to supplementation with vitamin D alone. The meta-analysis estimated the number needed to treat (NNT) an adverse outcome to be 276 (95% CI: 165-843) for hip fractures and 72 (95% CI: 35–834) for non-vertebral fractures (20).

Published estimates of the level of circulation 25(OH)D required to maintain normal levels of PTH range between 30 and 100 nmol/l (21). Low circulating levels of 25(OH)D increase secretion of parathyroid hormone (PTH) which in turn, induces bone loss in the elderly through increased bone resorption (22-24).

After vitamin D supplementation, we observed a significant reduction in pain intensity measured by VAS. After 6 months of vitamin D therapy, we detected a significant reduction in pain intensity. This suggests that vitamin D might improve the symptoms of this condition.

CONCLUSIONS

Supplementation with calcium and vitamin D can be justified in terms of both efficacy and health economics in women at increased risk of osteoporotic fracture, including those who have not yet sustained a fracture.

Women can be considered to be at increased risk of fracture if they are over 65 years of age, or if osteopenic and/or with proven calcium and/or vitamin D insufficiency when they are younger. Evidence suggests that for the greatest reduction in fracture risk, women at increased risk should be given both calcium and vitamin D supplements, in the order of 1000 mg calcium in alternate days (depending on baseline status) and 100000 IU vitamin D monthly. Patient adherence is essential for efficacy. Combining vitamin D and calcium into one supplement is recommended for all women at increased risk of fracture, including those also taking other anti-osteoporostic treatments.

Vitamin D supplementation significantly increased the 25(OH)D levels and caused a significant reduction in pain intensity in post-menopausal woman. This finding could suggest that vitamin D therapy may reduce pain intensity. A reduction in pain can lead to an improved quality of life. Further studies should address this topic.

REFERENCES

1. Marx SJ, Liberman UA, Eil C. Calciferols: actions and deficiencies in action. Vitamins & Hormones 1983; 40:235-308.
2. Costa EM, Blau HM, Feldman D. 1.25-Dihydroxyvitamin D3 receptors and hormonal responses in cloned human skeletal muscle cells. Endocrinology 1986; 119(5):2214-20.
3. Valtueña J, Gracia-Marco L, Huybrechts I, et al. Cardiorespiratory fitness in males, and upper limbs muscular strength in females, are positively related with 25-hydroxyvitamin D plasma concentrations in European adolescents: the HELENA Study. QJM 2013; 106(9):809-21.
4. Holick MF. Sunlight and vitamin D for bone health and prevention of autoimmune diseases, cancers, and cardiovascular disease. Am J Clin Nutr 2004; 80(6):1678S-88S.
5. Annweiler C, Allali G, Allain P, Bridenbaugh S, Schott AM, Kressig RW, Beauchet O. Vitamin D and cognitive performance in adults: a systematic review. Europ J Neurol 2009; 16(10)1083-9.
6. Yoshikawa S, Nakamura T, Tanabe H, Imamura T.

Osteomalacic myopathy. Endocrin Jap 1979; 26:65-72.
7. Adami S, Giannini S, Bianchi G, Sinigaglia L, Di Munno O, Fiore CE, Minisola S, Rossini M. Vitamin D status and response to treatment in postmenopausal osteoporosis. Osteoporosis Int 2009; 20:239-44.
8. Chel V, Wijnhoven HA, Smit JH, Ooms M, Lips P. Efficacy of different doses and time intervals of oral vitamin D supplementation with or without calcium in elderly nursing home residents. Osteoporos Int 2008; 19(5):663-71.
9. Ish-Shalom S, Segal E, Salganik T, Raz B, Bromberg IL, Vieth R. Comparison of daily, weekly and monthly vitamin D3 in ethanol dosing protocols for two months in elderly hip fracture patients. J Clin Endocrinol Metab 2008; 93(9):3430-5.
10. Bacon CJ, Gamble GD, Horne AM, Scott MA, Reid IR. High dose oral vitamin D3 supplementation in the elderly. Osteoporos Int 2009; 20(8):1407-15.
11. Von Restorff C, Bischoff-Ferrari HA, Theiler R. High dose oral vitamin D3 supplementation in rheumatology patients with severe vitamin D3 deficiency. Bone 2009; 45(4):747-9.
12. Hoeck HC, Li B, Qvist P. Changes in 25-hydroxyvitamin D3 to oral treatment with vitamin D3 in postmenopausal females with osteoporosis. Osteoporos Int 2009; 20:1329-35.
13. Vieth R. Vitamin D supplementation, 25-hydroxyvitamin D concentrations, and safety. Am J Clin Nutr 1999; 69(5):842-56.
14. Aloia JF, Patel M, DiMaano R, Li M, Talwar SA, Mikhail M, Pollack S, Yeh JK. Vitamin D intake to attain desired serum 25-hydroxyvitamin D concentration. Am J Clin Nutr 2008; 87(6):1952-8.
15. Bischoff-Ferrari HA, Willett WC, Wong JB, Giovannucci E, Dietrich T, Dawson-Hughes B. Fracture prevention with vitamin D supplementation. JAMA 2005; 293:2257-64.
16. Heikinheimo RJ, Inkovaara JA, Harju EJ, et al. Annual injection of vitamin D and fractures of aged bones. Calcif Tissue Int 1992; 51(2):105-10.
17. Bischoff-Ferrari HA. How to select the doses of vitamin D in the management of osteoporosis. Osteoporos Int 2007; 18(4):401-7.
18. Prince RL, Devine A, Dhaliwal SS, Dick IM. Effects of calcium supplementation on clinical fracture and bone structure: results of a 5-year, double-blind, placebo-controlled trial in elderly women. Arch Intern Med 2006; 166:869-75.
19. Shea B, Wells G, Cranney A, et al. Meta-analysis of calcium supplementation for the prevention of postmenopausal osteoporosis. Endocr Rev 2002; 23:552-9.
20. Boonen S1, Lips P, Bouillon R, Bischoff-Ferrari HA, Vanderschueren D, Haentjens P. Need for additional calcium to reduce the risk of hip fracture with vitamin D supplementation: evidence from a comparative meta-analysis of randomized controlled trials. J Clin Endocrinol Metab 2007; 92:1415-23.
21. Dawson-Hughes B, Heaney RP, Holick MF, Lips P, Meunier PJ, Vieth R. Estimates of optimal vitamin D status. Osteoporos Int 2005; 16:713-6.
22. Boonen S, Lips P, Bouillon R, Bischoff-Ferrari HA, Vanderschueren D, Haentjens P. Need for additional calcium to reduce the risk of hip fracture with vitamin D supplementation: evidence from a comparative meta-analysis of randomized controlled trials. J Clin Endocrinol Metab 2007; 92:1415-23.
23. Chapuy MC, Pamphile R, Paris E, Kempf C, Schlichting M, Arnaud S, Garnero P, Meunier PJ. Combined calcium and vitamin D3 supplementation in elderly women: confirmation of reversal of secondary hyperparathyroidism and hip fracture risk: the Decalyos II study. Osteoporos Int 2002; 13:257-64.
24. Sahota O, Mundey MK, San P, Godber IM, Lawson N, Hosking DJ. The relationship between vitamin D and parathyroid hormone: calcium homeostasis, bone turnover, and bone mineral density in postmenopausal women with established osteoporosis. Bone 2004; 35:312-9.

OPTIMAL IMPROVEMENT IN FUNCTION AFTER TOTAL HIP AND KNEE REPLACEMENT: HOW DEEP DO YOU KNOW YOUR PATIENT'S MIND?

M. F. DE CARO[1], G. VICENTI[2], A. ABATE[3], G. PICCA[2], V. LEONCINI[1], M. LOMUSCIO[1], A. CASALINO[2], G. SOLARINO[2] AND B. MORETTI[2]

[1]Department of Neuroscience and Organs of Sense, Psychology section, Faculty of Medicine and Surgery, University of Bari, Bari, Italy; [2]Department of Neuroscience and Organs of Sense, Orthopedics Section, Faculty of Medicine and Surgery, University of Bari, Bari, Italy; [3]Department of Orthopedics and Traumatology, Monsignor Raffaele Dimiccoli Hospital, Barletta, Italy

Osteoarthritis (OA) of the hip and knee causes pain and loss of joint mobility, leading to limitations in physical function. When conservative treatment fails total hip and knee replacement is a cost-effective surgical option. Patients have high expectations regarding functional outcome after these procedures. If such expectations are not met, they may still be dissatisfied with the outcome of a technically successful procedure. Recently, numerous studies reported that psychological factors can influence the outcome of total knee replacement (tkr) and total hip artrhoplasty with total hip replacement (thr). We conducted a prospective study on a consecutive sample of 280 patients affected by hip or knee OA who underwent total joint replacement. At patients' admission, Harris Hip Score (HHS) and Knee Society Score (KSS) were used to assess pain and function. Furthermore, SF-36, Mini-Mental Status Examination (MMSE), Symptom Checklist-90-R (SCL-90-R), Coping Orientation to Problems Experienced (BRIEF-COPE) and the Amsterdam Preoperative Anxiety and Information Scale (APAIS) were administered. Patients had clinical and radio graphical follow up at 1, 3 and 6 months post-operatively. The HHS and KSS values before surgery showed a linear correlation with both SCL-90-R and MMSE. None of the investigated variables influenced post-operative HHS and KSS scores; however, the improvement of functional scores resulted conditioned by SCL-90-R values, VAS score, schooling and MMSE. Psychological factors and mental status in primary total hip and knee replacement can affect outcome and patient satisfaction. Strategies focused on identification and facing of these conditions must be considered to improve outcome of total replacement.

When conservative treatment fails to reduce knee or hip pain and functional activity, total knee replacement and total hip replacement are considered cost-effective surgical options for patients affected by end stage osteoarthritis (1223 patients who were enrolled for hip or knee replacement surgery were asked to fill in the 15D health-related quality of life.

Additionally, the number of these surgical procedures is constantly rising due to the increase in average life expectancy. TKR and THR are actually one of the most performed surgeries worldwide The aims of these procedures are pain and disability resolution to improve quality of life.

However some patients experience moderate to severe level of persistent post-operative pain after surgery, despite an evidence of both clinical and

Key words: total knee replacement, total hip replacement, outcomes, psychological factors, complications

Mailing address: Giovanni Vicenti, MD,
Department of Neuroscience and Organs of Sense,
Orthopedics Section, Faculty of Medicine and Surgery,
University of Bari, Policlinico,
Piazza Giulio Cesare 11, 70124 Bari, (BA), Italy
Tel.: +393480128360
e-mail: dott.gvicenti@gmail.com

0393-974X (2015)

radiological abnormalities (5-7). Additionally there is often a disparity between patient and surgeon perception of outcome. Moreover, surgeons ignore patient perception of residual dissatisfaction because their evaluation is strictly based on objective results.

This lack of concordance is the results of an initial misunderstanding between surgeon and patients, secondary to the lack of active dialogue, clear comprehension of patient expectations, and univocal decision-making process before surgical procedure. Empathic approach on behalf of the surgeon should improve patient selection before surgery and provide a pre-operative clinical and welfare terrain necessary to obtain satisfactory outcomes according to the patient.

Psychological factors such as mental health, patient expectation, anxiety and depression influence outcome of replacement surgery (8). Numerous studies have reported that psychological factors can influence the outcome of total knee arthroplasty. Furthermore, pre-surgical mental status and level of catastrophizing have been reported as the most important predictors of pain after THR and TKR.

Patients suffering from chronic medical illnesses, like those affected by end stage OA, show an high prevalence of psychological symptoms which influence their motivation, energy, their ability to cope with the illness and the amount of adherence to rehabilitative protocols (13).

During the last several years a number of studies have reported that patients with psychological symptoms have a worse outcome after TKR or THR (8). Vissers, et al. showed that patients with a preoperative lower mental health score were more often dissatisfied with results after TKR (14).

In the present study, patients scheduled for THR and TKR were assessed between 1 to 3 months before surgery and then again after 6 months post-surgery. We examined the prevalence of anxiety and depressive symptoms in patients with end stage OA and then we investigated the relationship between the psychological symptoms and surgical outcome.

MATERIALS AND METHODS

This was a prospective study conducted at the University Hospital, Policlinico Bari between April 2011 and November 2014. A consecutive sample of 280 patients with hip or knee OA were enrolled in the study. Ethical approval was granted and all patients provided their written informed consent to participate in the study. Inclusion criteria were patients aged between 45 and 75, being able to understand written information (informed consent) and free from any psychiatric or neurologic pathology. Patients who had more than 2 comorbidities as well as patients who had undergone surgery for fractures or previous implant failure were excluded from the study.

Patients were invited to take part to the study when they came to the hospital for routine pre-operative exams, 1 to 3 months before surgery. If a patient was considered eligible and signed the consent, an orthopedic resident not involved in the surgery performed a clinical objective evaluation. A psychologist then conducted an interview.

Pain and Function

The Harris Hip Score (HHS) and Knee Society Score (KSS) were used to assess pain and function. HHS was developed for the assessment of the results of hip surgery and is intended to evaluate various hip disabilities and methods of treatment in an adult population. The domains covered are pain, function, absence of deformity, and range of motion. It consists of 10 items with a maximum score of 100 points (best possible outcome).

The Knee Society Score is reported as two scores, Knee Score and Function Score. The Knee Score consists of points given for pain, range of motion and stability. The Function Score consists of points given for the ability to walk on level surfaces and the ability to ascend and descend stairs. The maximum of 100 points is obtained by a well-aligned knee with no pain (knee score) and by a person who can walk an unlimited distance and go up and down stairs (function score).

Socio-Dermographic:

It included questions on age, schooling, residence, marital status, professional status, household and parity.

MMSE:

The Mini-Mental Status Examination offers a quick and simple way to quantify cognitive function and screen for cognitive loss. It tests individual orientation, attention, calculation, recall, language and motor skills. Each section of the test involves a related series of questions or commands. The individual receives one point for each correct answer. The maximum score is 30 points. A score below 20 usually indicates cognitive impairment.

SCL-90:

The Symptom Checklist-90-R (SCL-90-R) is a relatively brief self-report psychometric instrument (questionnaire) published by the Clinical Assessment division of the Pearson Assessment & Information group.

It is designed to evaluate a broad range of psychological problems and symptoms of psychopathology. It is also used in measuring the progress and outcome of psychiatric and psychological treatments or for research purposes. It consists of 90 items and takes 12-15 minutes to administer, yielding nine scores along primary symptom dimensions and three scores among global distress indices.

The primary symptom dimensions that are assessed are somatization, obsessive-compulsive, interpersonal sensitivity, depression, anxiety, hostility, phobic anxiety, paranoid ideation, psychoticism, and a category of "additional items" which helps clinicians assess other aspect of the client's symptoms.

BRIEF-COPE: The Coping Orientation to Problems Experienced is a multi-dimensional scale designed to assess how people respond to stress. The COPE has been validated in a variety of populations displaying variations in factor structure. The Brief COPE (Carver, 1997) is a self-report questionnaire used to assess a number of different coping behaviors and thoughts a person may have in response to a specific situation. It contains 14 subscales, but we analyzed only two of those: use of social support and religion.

APAIS: In 1996 the Dutch group of Moerman developed the Amsterdam Preoperative Anxiety and Information Scale APAIS 15320 patients were asked to assess their anxiety and information requirement on a six-item questionnaire, the Amsterdam Preoperative Anxiety and Information Scale (APAIS. This questionnaire consists of six items rated on a five point Likert scale with the end poles "not at all" (1) and "extremely" (5). It represents two scales, anxiety and need-for-information.

SF-36: SF-36 is a set of generic, coherent, and easily administered quality-of-life measures. These measures rely upon patient self-reporting and now widely utilized by managed care organizations and by Medicare for routine monitoring and assessment of care outcomes in adult patients. It contains 36 questions, which measure functional health and well-being from the patient's point of view. It is a practical, reliable and valid measure of physical and mental health and can be used as alternative to disease-specific health surveys, which focus on a particular condition or disease.

Procedure: Patients who were scheduled for a THR or TKR at our hospital were invited to participate in the research and were informed about the aim of the research. Psychological evaluation was taken before surgery, while pre- and post-operatively clinical and radiographic exams were carried out at 1, 3 and 6 months. Informed consent was obtained. The Research Ethic Board of our Centre approved the research. Participants were evaluated and interviewed at the time of their pre-surgical evaluation (between 1 and 3 months before surgery). Surgery was performed by 4 experienced surgeons. One-hundred-and-ninety-five patients underwent spinal anesthesia and 85 general anesthesia. They all received the same protocol for post-operative pain management. All surgeons used the same approach to surgery. Patients received short-term systemic antibiotics and Low Molecular Weight heparin to decrease the risk of deep venous thrombosis. Negative pressure drainage was positioned and removed 24 h after surgery. Post-operative X-rays were taken and patients were treated with the same rehabilitation protocol for the 3 weeks following.

Statistics: The data analysis was conducted using regression models to assess:

– The association between the Socio-Dermographic variables, MMSE, SCL-90, BRIEF-COPE, APAIS, SF-36 and hip and knee functional scales before surgery.

– The association between the Socio-Dermographic variables, MMSE, SCL-90, BRIEF-COPE, APAIS, SF-36 and the improvement in hip and knee function after surgery.

We used Statistical Package for the Social Sciences (SPSS) version 19.0 (Chicago, IL) to perform analyses.

RESULTS

Between April 2011 and November 2014, 412 patients were eligible to participate in the study. Only 280 patients accepted and received preoperative questionnaire. No patients were lost during follow up (Fig. 1). Table I presents the baseline characteristics of the study population.

Before surgery, the average value of the HHS was 36.5±1.0; the average KSS score was 49.0±1.0. After surgery, the average value of the HHS was 82.8±5.2; the average KSS score was 82.9±7.9. All patients showed an improvement in the HHS and KSS after surgery (Table II).

The HHS and KSS values before surgery had a linear correlation with the sc90som (r2=0:13; F=5:26; p=0.03), the scl90dep (r2=0.12 F=4.54; F=0.04), the mmsec (r2=0.16; F=7.7; p=0.009).

None of the investigated variables influenced post-operative HHS and KSS scores, however, the improvement of functional scores were conditioned by the sc90som (coef=0:32; t=2.46; p=0.026), vas (coef=-0.15, t=2.57, p=0.021), schooling (coef=1.8; t=2.93; p=0.01) and MMSE (coef=1.94, t=3.28; p=0.005). Coping strategies did not correlate to the level of improvement after surgery.

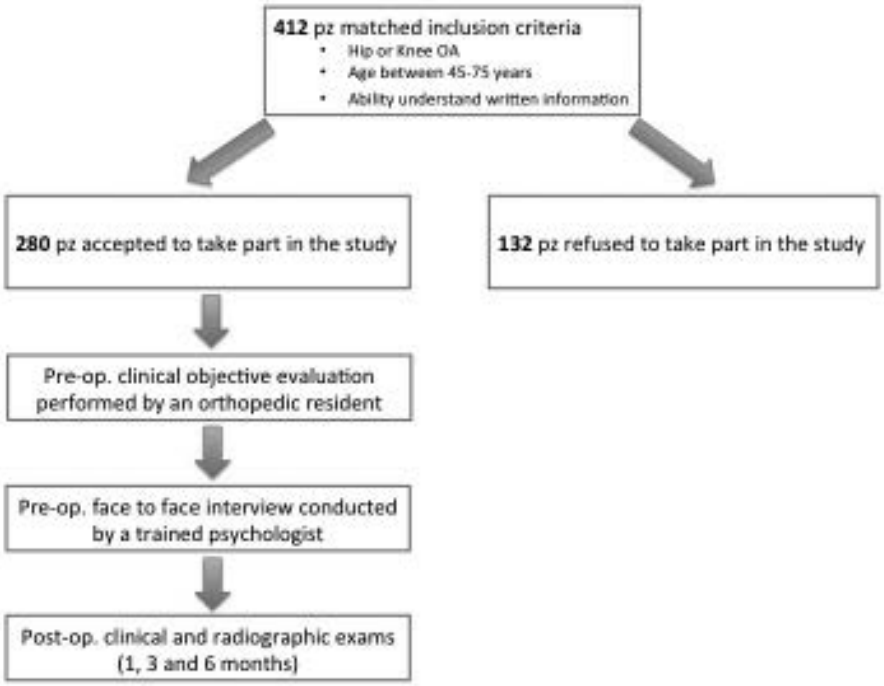


Fig. 1. *Procedure of Selection Flowchart.*

Table I. *Baseline characteristics of the study population.*

VARIABLES	
Age	45-75 (Mean 64,6)
Gender	78% FEMALE
Education (schooling)	38% PRIMARY SCHOOL 41% SECONDARY SCHOOL 21% GRADUATED
SCL-90 SOM	52.6±21.9
SCL-90 DEP	50.7±20.1
SCL-90 DEP	45.7±18.0
VAS	51.5±31.0
APAIS-ANX	9.1±4.3
BRIEF-COPE-SOC	7.9±5.1
BRIEF-COPE-REL	4.4±1.9
SF-36	58.9±20.3
MMSE	27.9±2.3

DISCUSSION

A high prevalence of anxiety and depressive symptoms were found in patients with hip and knee OA. Perception of health in preoperative patients (SF-36) did not correlate with psychological factors confirmed by the SF 36 questionnaire (8).

In this study, we found that psychological

Table II. *Pre- and post-operative HHS and KSS values.*

	PRE-OPERATIVE	POST-OPERATIVE
HARRIS HIP SCORE (HHS)	36.5 ± 1.0	82.8 ± 5.2
KNEE SOCIETY SCORE (KSS)	49.0 ± 1.0	82.9 ± 7.9

factors (som SCL-90), mental health status, VAS and scholarity were associated with less optimal improvement in function after total hip and knee replacement. This confirms available evidence about the influence of psychological factors on TKR and THR outcomes (8). All these conditions can invalidate successful post-operative rehabilitation program and compromise the perception of health (16, 17). Somatic symptom disorder is a long-term (chronic) condition able to influence intensity and perception of pain and functional limitation. High levels of somatization might influence the neurophysiological process involved in pain modulation (18). Additionally, pain somatization can determine a prolonged period of inactivity and compounding disability secondary to comorbidities development (obesity, diabetes) (19).

Studies suggest that almost 50% of individuals with post-operative pain at 6 months after TJR might go on to develop chronic pain condition (20-22). At this stage chronic pain could be hard to manage successfully either through pharmacological or psychological methods (23-25). This can contribute to a progressive decline towards a sedentary lifestyle and social isolation (26, 27). Additionally, the coping ability represents a potentially modifiable variable and could be the focus of a pre-operative psychological intervention (28). Our study did not support the association between coping ability and level of improvement after surgery.

If individuals that are at risk in unsatisfactory joint replacement can be indentified before surgery takes place, their suffering could be prevented or reduced, avoiding chronic pain and disability. Measures could be taken before and after surgery with dedicated psychological tutoring and rehabilitation protocols. Furthermore, surgery could be delayed if the state of mental health is in a better condition.

Educating pre-operative patients through instructing and training on standard rehabilitative exercises can be applied routinely so that even patients with minor values of MMES and mental disorders (depression and anxiety) can be considered for targeted interventions. According to our study, schooling represents a significant correlation with improved outcome. Papakostiodu, et al. found that level of schooling did not interfere with post-surgical quality of life levels (29). Although no comparative studies have yet been conducted, available data suggest that cognitive and behavioral techniques and their comprehension by patients might be effective in reducing pain related fear of movement (30, 31).

Preventive psychosocial interventions for OA patients have primarily taken the form of broad-based educational programs. Since education programs do not select individuals at risk, nor do they target specific prognostic factors for pain and disabilities, it is not surprising that they have yielded only modest impact on surgical outcomes (32).

Depression may contribute to poor TKR outcomes either in terms of pain, functions and quality of life symptoms (23, 33), however Sullivan, et al. stated that depression shouldn't be considered a unique contribution to problematic health outcomes after replacement (34).

We did not find a direct correlation between clinical outcomes and pre-operative anxiety and depression, however these symptoms showed a linear correlation with the post-operative entity

of functional improvement. Most studies on post-operative pain have been short term while longer follow up periods might be as long as two years. Since psychological factors are likely to have a dynamic correlation with the changing health status over time, longer follow-up periods might be needed to provide reliable results.

Sepucha, et al. stated that an open discussion about the nature of OA and surgical procedure between the patient and the healthcare provider might improve the outcome (35). Braddock, et al. found that an open discussion of the informed decision-making process happens in fewer than 15% of cases (36). Patient communication and explanation of goals is a key part of the decision-making process but it is often neglected by surgeons (37).

We investigated the need for information on behalf of the patient (APAIS-INFO) and we found no correlation with surgical outcomes. Careful information is standardly provided with pre-operative meetings at our department. Groups of 6 to 7 patients and their caregivers are invited to participate at the meetings to obtain information on surgical procedures, goals of the treatment and the post-operative period. We strongly believe this can help patient awareness of treatments and improve outcome.

Recent health care legislation reiterates the importance in order to ensure that both the patient and its family are informed during the medical decisional process. Shared decision-making is associated with improvement in clinical outcome and reduce social burdens of surgical procedures. Patient awareness of surgical procedures improves knowledge of goals of treatments, reducing breakdown and dissatisfaction (35, 38).

Correct patient selection in association with setting appropriated expectations, avoiding preventable complications and optimizing pre- and post-operative pathways may enhance patients outcomes (39).

The Strength of our study is in its prospective design, the use of validated questionnaires and their results. However, some limitations need to be addressed. We did not divide our considerations between patients affected by hip or knee OA, however a recent review asserts that differences between knee and hip patients could be explained by the relatively few studies that evaluated influence of psychological factors affecting outcome after THR8. We therefore decided to combine the results of THR and TKR. Preoperative measurements were not taken at a fixed time point and the length of time patients were on the waiting list varied. Desmueles, et al. observed that a long wait for surgery had a significantly negative impact on pain, function and quality of life (40); McHugh, et al. observed a worsening of pain and function on the WOMAC scale starting from the third month of waiting (41). The use of questionnaires at different times before surgery might be interesting in order to compare distinct results.

We did not evaluate the influence of comorbidities which might determine higher post-operative complication rates which influence post-operative adherence to rehabilitation programs and delay functional results. However, patients with major comorbidities were excluded from the study at the time of enrollment. Our mean follow up consists of 6 months. Longer follow-ups are necessary especially for patients with chronic diseases, which might invalidate functional replacement results after a longer period.

REFERENCES

1. Räsänen P, Paavolainen P, Sintonen H, Koivisto AM, Blom M, Ryynänen OP, Roine RP. Effectiveness of hip or knee replacement surgery in terms of quality-adjusted life years and costs. Acta Orthop 2007; 78(1):108-15.
2. Kurtz S, Ong K, Lau E, Mowat F, Halpern M. Projections of primary and revision hip and knee arthroplasty in the United States from 2005 to 2030. J Bone Joint Surg Am 2007; 89:780-5.
3. Learmonth ID, Young C, Rorabeck C. The operation of the century: total hip replacement. Lancet 2007; 370:1508-19.
4. Losina E1, Walensky RP, Kessler CL. Cost-effectiveness of total knee arthroplasty in the United States: patient risk and hospital volume. Arch Intern Med 2009; 169:1113-21.
5. Brander VA, Stulberg SD, Adams AD, Harden RN, Bruehl S, Stanos SP, Houle T. Predicting total knee replacement pain: a prospective, observational study. Clin Orthop Relat Res 2003; 27-36.
6. Jones CA, Voaklander DC, Johnston DW, Suarez-

Almazor ME. Health related quality of life outcomes after total hip and knee arthroplasties in a community based population. J Rheumatol 2000; 27:1745-52.
7. Wylde V, Dieppe P, Hewlett S, Learmonth ID. Total knee replacement: is it really an effective procedure for all? Knee 2007; 14:417-23.
8. Vissers MM, Bussmann JB, Verhaar JA, Busschbach JJ, Bierma-Zeinstra SM, Reijman M. Psychological factors affecting the outcome of total hip and knee arthroplasty: A systematic review. Semin Arthritis Rheum 2012; 41:576-88.
9. Wylde V, Hewlett S, Learmonth ID, Dieppe P. Persistent pain after joint replacement: prevalence, sensory qualities, and postoperative determinants. Pain 2011: 152:566-72.
10. Faller H, Kirschner S, König A. Psychological distress predicts functional outcomes at three and twelve months after total knee arthroplasty. Gen Hosp Psychiatry 2003; 25:372-3.
11. Forsythe ME1, Dunbar MJ, Hennigar AW, Sullivan MJ, Gross M. Prospective relation between catastrophizing and residual pain following knee arthroplasty: two-year follow-up. Pain Res Manag 2008; 13:335–41.
12. Sullivan M, Tanzer M, Reardon G, Amirault D, Dunbar M, Stanish W. The role of presurgical expectancies in predicting pain and function one year following total knee arthroplasty. Pain 2011; 152:2287-93.
13. Dikmans-Vissers MM. Recovery after total hip or knee arthroplasty: physical and mental functioning. Int J Pediatr (2012).
14. Vissers MM, de Groot IB, Reijman M, Bussmann JB, Stam HJ, Verhaar JA. Functional capacity and actual daily activity do not contribute to patient satisfaction after total knee arthroplasty. BMC Musculoskelet Disord 2010; 11**:**121.
15. Moerman N, van Dam FS, Muller MJ, Oosting H. The Amsterdam Preoperative Anxiety and Information Scale (APAIS). Anesth Analg 1996; 82:445-51.
16. McGrady A, McGinnis R, Badenhop D, Bentle M, Rajput M. Effects of depression and anxiety on adherence to cardiac rehabilitation. J Cardiopulm Rehabil Prev 2009; 29:358-64.
17. Glazer KM, Emery CF, Frid DJ, Banyasz RE. Psychological predictors of adherence and outcomes among patients in cardiac rehabilitation. J Cardiopulm Rehabil 2002; 22:40-6.
18. Fillingim RB, Hastie BA, Ness TJ, Glover TL, Campbell CM, Staud R. Sex-related psychological predictors of baseline pain perception and analgesic responses to pentazocine. Biol Psychol 2005; 69:97-112.
19. Heuts PH, Vlaeyen JW, Roelofs J, de Bie RA, Aretz K, van Weel C, van Schayck OC. Pain-related fear and daily functioning in patients with osteoarthritis. Pain 2004; 110:228-35.
20. Harden RN, Bruehl S, Stanos S, Brander V, Chung OY, Saltz S, Adams A, Stulberg SD. Prospective examination of pain-related and psychological predictors of CRPS-like phenomena following total knee arthroplasty: a preliminary study. Pain 2003; 106:393-400.
21. Kennedy DM, Hanna SE, Stratford PW, Wessel J, Gollish JD. Preoperative function and gender predict pattern of functional recovery after hip and knee arthroplasty. J Arthroplasty 2006; 21:559-66.
22. Kennedy DM, Stratford PW, Hanna SE, Wessel J, Gollish JD. Modeling early recovery of physical function following hip and knee arthroplasty. BMC Musculoskelet Disord 2006; 7:100.
23. Fortin PR, Clarke AE, Joseph L. Outcomes of total hip and knee replacement: preoperative functional status predicts outcomes at six months after surgery. Arthritis Rheum 1999; 42: 1722-8.
24. McAlindon TE, Cooper C, Kirwan JR, Dieppe PA. Determinants of disability in osteoarthritis of the knee. Ann Rheum Dis 1993; 52:258-62.
25. van Baar ME, Dekker J, Lemmens JA, Oostendorp RA, Bijlsma JW. Pain and disability in patients with osteoarthritis of hip or knee: the relationship with articular, kinesiological, and psychological characteristics. J Rheumatol 1998; 25:125-3.
26. Campbell LC, Clauw DJ, Keefe FJ. Persistent pain and depression: a biopsychosocial perspective. Biol Psychiatry 2003; 54:399-409.
27. Keefe FJ, Caldwell DS, Martinez S, Nunley J, Beckham J, Williams DA. Analyzing pain in rheumatoid arthritis patients. Pain coping strategies in patients who have had knee replacement surgery. Pain 1991; 46:153-60.
28. Kendell K, Saxby B, Farrow M, Naisby C.

Psychological factors associated with short-term recovery from total knee replacement. Br J Health Psychol 2001; 6:41-52.
29. Papakostidou I, Dailiana ZH, Papapolychroniou T, Liaropoulos L, Zintzaras E, Karachalios TS, Malizos KN. Factors affecting the quality of life after total knee arthroplasties: a prospective study. BMC Musculoskelet Disord 2012; 13:116.
30. Thorn BE, Pence LB, Ward LC, Kilgo G, Clements KL, Cross TH, Davis AM, Tsui PW. A randomized clinical trial of targeted cognitive behavioral treatment to reduce catastrophizing in chronic headache sufferers. J Pain 2007; 8(12):938-49.
31. Woods MP, Asmundson GJ. Evaluating the efficacy of graded in vivo exposure for the treatment of fear in patients with chronic back pain: a randomized controlled clinical trial. Pain. 2008; 136(3):271-80.
32. McDonald S, Hetrick S, Green S. Pre-operative education for hip or knee replacement. Cochrane Database Syst Rev 2004; (1):CD003526.
33. Fisher DA, Dierckman B, Watts MR, Davis K. Looks good but feels bad: factors that contribute to poor results after total knee arthroplasty. J Arthroplasty.2007; 22(6 Suppl 2):39-42.
34. Sullivan M, Tanzer M, Stanish W, Fallaha M, Keefe FJ, Simmonds M, Dunbar M. Psychological determinants of problematic outcomes following Total Knee Arthroplasty. Pain 2009; 143(1-2):123-9.
35. Sepucha K, Feibelmann S, Chang Y, Clay CF, Kearing SA, Tomek I, Yang T, Katz JN. Factors associated with the quality of patients' surgical decisions for treatment of hip and knee osteoarthritis. J Am Coll Surg 2013; 217(4):694-701.
36. Braddock C 3rd, Hudak PL, Feldman JJ, Bereknyei S, Frankel RM, Levinson W. 'Surgery is certainly one good option': quality and time-efficiency of informed decision-making in surgery. J Bone Joint Surg Am 2008; 90(9):1830-8.
37. Weinstein JN, Clay K, Morgan TS. Informed patient choice: patient-centered valuing of surgical risks and benefits. Health Aff (Millwood) 2007; 26(3):726-30.
38. American Institutes for Research. Shared-Decision-Making and Benefit Design; Engaging Employees and Reducing Costs for Preference-Sensitive Conditions. Robert Wood Johnson Found 2013.
39. Drexler M, Dwyer T, Chakravertty R, Farno A, Backstein D. Assuring the happy total knee replacement patient. Rheumatology (Oxford) 2010; 49(5):945-54.
40. Desmeules F, Dionne CE, Belzile E, Bourbonnais R, Frémont P. The burden of wait for knee replacement surgery: effects on pain, function and health-related quality of life at the time of surgery. Rheumatology (Oxford) 2010; 49(5):945-54.
41. McHugh GA1, Luker KA, Campbell M, Kay PR, Silman AJ. Pain, physical functioning and quality of life of individuals awaiting total joint replacement: a longitudinal study. J Eval Clin Pract 2008; 14(1):19-26.

ANTIBIOTIC-LOADED REGENOSS FOR THE TREATMENT OF SEPTIC BONE DEFECTS: *IN VITRO* STUDY AND PRELIMINARY CLINICAL EXPERIENCE

C.L. ROMANÒ[1], E. GALLAZZI[1], N. LOGOLUSO[1]. E. DE VECCHI[2], L. DRAGO[2]

[1]Department of Reconstructive Surgery of Osteo-articular Infections C.R.I.O. Unit, I.R.C.C.S. Galeazzi Orthopaedic Institute, Milano, Italy; [2]Laboratory of Clinical Chemistry and Microbiology, I.R.C.C.S. Galeazzi Orthopaedic Institute, Milano, Italy

Bone and joint infections are a difficult to treat condition, often associated with bone loss. Although the management of septic bone defects may currently be achieved through various treatment modalities, there is a continuous need for bone substitutes able at the same time to favour bone repair and to provide local antibacterial protection. RegenOss, a biomimetic and resorbable bone substitute, has been previously shown to be highly biocompatible and osteoconductive. Aims of the present study were to test the *in vitro* ability of RegenOss to act as a local carrier of antibiotics and to investigate its clinical safety and efficacy in a continuous series of patients, affected by bone loss in active or previous infection. *In vitro* study was performed by adding vancomycin, levofloxacin or meropenem and assessing elution properties of RegenOss at fixed time intervals by means of a microbiological assay. At 48 hours, 98.5% of meropenem, 94.1% of levofloxacin and 76.3% of vancomycin were recovered in the medium, while all antibiotics were completely eluted at seven days. Clinical safety and efficacy of vancomicyn- or vancomicyn and meropenem-loaded RegenOss had been tested in 13 consecutive patients. After the surgical procedure, each patient underwent clinical, laboratory and radiographic evaluation at 3, 6, 12, 18 and 24 months. No adverse events associated with the use of RegenOss were observed. Twelve patients showed no infection recurrence and ten satisfactory bone healing at follow-up. In conclusion, this study shows the ability of RegenOss to act as local carrier when loaded with three different antibiotics with a complete elution in one week. The clinical use of antibiotic-loaded RegenOss appears safe in this preliminary clinical series, while larger studies are needed to confirm the efficacy of the intra-operative combination of this biomimetic bone substitute with various antibacterials in the treatment of septic bone defects.

Bone and joint infections (BJIs) still remain a major complication in orthopaedic and trauma surgery (1). Bone infection can in fact be the result of direct bacterial penetration into the bone or the adjacent soft tissues following surgical procedures or after low or high-energy trauma. The presence of implanted biomaterials or foreign bodies strongly increase the risk of septic complications, due to the ability of bacteria to adhere on implant surfaces and to form protective biofilms (2-4). Less frequently, at least in the most developed Countries, osteomyelitis can originate from haematogenous spreading of the microorganisms from other sites.

Virulence of the causative microorganisms and their antibiotic sensitivity, timing of treatment, as well as host's risk factors, like diabetes, renal impairment, smoking, drug or alcohol abuse, etc. (3, 5), play an important role in the clinical presentation,

Key words: orthopaedics, trauma, implant, joint, prosthesis, infection, bone defect, bone substitute, RegenOss

Mailing address: Dr C. L. Romanò,
Department of Reconstructive Surgery of Osteo-articular Infections,
IRCCS Galeazzi Orthopaedic Institute,
Via R. Galeazzi 4 20161 Milan, Italy,
Tel.: +39 02 66214903 Fax: +39 02 66214774
e-mail: carlo.romano@grupposandonato.it

0393-974X (2015)

prognosis and outcomes of treatment of BJIs (1). *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Escherichia coli* and *Pseudomonas aeruginosa* are among the most commonly involved pathogens in BJIs (6-8), often leading to a chronic inflammatory disease and to the formation of necrotic bone (1), which generally require large surgical resections and prolonged antibiotic therapy.

The septic process *per se,* the frequent occurrence of previous trauma or surgeries and the need for further bone resection do often lead to various degrees of bone loss and impaired local blood supply, which in turn may reduce the efficacy of systemic antibiotic treatments (9).

In this complex scenario, effective infection control and bone defect management often need to be combined in order to successfully treat chronic BJIs and their sequelae.

Autologous bone graft is considered the "golden standard" to fill bone defects and to achieve bone repair. However, donor side morbidity, chronic pain and infection at the harvesting site (10-12), as well limited availability prevent its routine use. On the other hand, synthetic bone substitutes may represent a valid alternative in the majority of cases (10) and new generation bone substitutes are increasingly used not only to fill bone defects, but also to foster bone regeneration (13). In addition, some of these devices have shown the ability to act as local carriers of antibiotics, thus acting at the same time as bone void filler, osteoconductive biomaterials and local antibacterials, minimizing the risk of systemic side effects and of possible lack of therapeutic concentrations in the infected area (14-16).

RegenOss, an approved medical device available in European countries, is a resorbable, biomimetic bone substitute, composed of equine collagen I and Mg-hydroxyapatite (ratio: 40%-60%), with chemical, chemico-physical and biological properties close to those of the mineral component of human bone (17). The patented nucleation of magnesium-substituting calcium hydroxyapatite nanocrystals into type I collagen fibers resembles the process occurring during biological neo-ossification, favouring cell attachment and proliferation and serving as a scaffold to guide effective bone regeneration (18), thus providing a reduction of the time to osteointegration, with minimal or no local side effects (11).

At variance with pre-loaded antibiotic-scaffolds currently available for the treatment of bone infections, that may not offer the choice of the antibiotic to be administered locally (19), we investigated *in vitro* and in the clinical setting the ability of RegenOss to act as a carrier of different antibiotics, chosen at the time of surgery and intra-operatively loaded by the surgeon.

MATERIALS AND METHODS

In vitro testing

To evaluate the release of antibiotics, strips of 1 cm^2 of RegenOss (Finceramica SpA, Faenza, Italy) were immersed in 0.35 mL of a 0.05 g/L solution of vancomycin, levofloxacin or meropenem for 10 min and then left at ambient air until complete drying. Then samples were transferred in tubes containing 5 mL of sterile phosphate buffered saline (PBS) and incubated at 37°C for one week. At 2, 16, 24, 48, 60, 96 hours and at the end of incubation (168 h), the eluate was refreshed with the same volume of PBS and antibiotic concentrations assessed by a microbiological assay using *Staphylococcus aureus* ATCC 25923 as indicator organism. The test was based on the diffusion of 200 µL aliquots of the PBS eluate from 6 mm wells into Mueller Hinton agar plates previously seeded with *S. aureus*. Plates were then incubated for 24 h at 37°C and inhibition zones were accurately measured. Calculation of antibiotic concentration at each time point was performed against standard curve covering a range of antibiotic concentrations from 0.01 to 6.25 mg/mL.

Samples containing antibiotics at concentrations near the upper limit of the standard curve were re-assessed after proper dilution in PBS. All samples were assessed in triplicate and the arithmetic means of the three test were used.

Clinical use of RegenOss

From November 2011 to March 2013, 13 consecutive patients (6 men, 7 women; mean age 55 years old, range 16-78 years) were included in this prospective, observational, not randomized study, approved by the local Ethical Committee. All patients gave their informed consent to the procedures.

The patients were selected among those who attended our clinic and had a planned surgical procedure in which there was the need for the treatment of cavitary or segmental bone defect in an active or previous bone infection. The selected patients covered a broad spectrum of pathologies, including septic non-union, chronic osteomyelitis and peri-prosthetic joint infection. Most of

the patients were affected by severe co-morbidities and had previously undergone several failed surgeries (Table I).

RegenOss was intra-operatively loaded either with vancomycin (N=8) or a combination of vancomycin-meropenem (N=5) (Table II). To this aim, the RegenOss patch was immersed in a solution containing 5% of the chosen antibiotic(s) and left in place for 10 min. In 4 patients antibiotic-loaded RegenOss had been used in combination with autologous or homologous morcellized bone grafts at a 50% weight-to-weight ratio. External fixation or implants were used as needed in 10 patients (Table II).

Follow-up consisted in clinical, laboratory and radiographic examination at approximately (+-20 days) 3, 6, 12, 18 and 24 months after surgery and yearly thereafter.

Recurrence of infection, defined as the presence of clinical signs of inflammation at the surgical site and an elevation of CRP and ESR serum levels was recorded.

Radiographic bone healing (filling of the bone gap or defect with new bone visible at x-ray examination) and the need for unplanned further surgery were also monitored at follow-ups, together with any local or systemic clinical side effect.

Table I. *Pre-operative data of patients treated with antibiotic-loaded RegenOss*

Initials	Sex	Age (years)	Relevant co-morbidities	Previous surgeries	Diagnosis
BM	M	40	Acquired immunodeficiency syndrome (AIDS)	2	Tibia septic non union
MS	F	16	None	0	Haematogenous osteomyelitis
AG	F	51	Smoker	3	Tibia septic incomplete union
PR	M	35	Smoker	3	Post-traumatic osteomyelitis of the tibia
PG	F	71	Rheumatoid arthritis	5	Peri-prosthetic hip infection (2nd stage)
DBS	F	52	Renal transplant	5	Peri-prosthetic hip infection (2nd stage)
PE	F	78	Old age	4	Tibia septic non union
GG	M	65	None	4	Peri-prosthetic hip infection (2nd stage)
ZM	M	70	Smoker, obese, pancreatic disorder	5	Septic nonunion in ankle arthrodesis
CM	M	75	None	4	Peri-prosthetic hip infection (2nd stage)
PT	F	72	Severe rheumatoid arthritis, corticosteroids, soft tissue defect	6	Peri-prosthetic femoral fracture in previous infection in revision knee prosthesis
CMT	F	60	Rheumatoid arthritis	3	Septic nonunion in ankle arthrodesis
SB	M	35	None	2	Chronic osteomyelitis of the tibia after anterio cruciate ligament reconstruction

Table II. *Intra and post-operative data.*

Initials	Treatment modality		Follow-up (months)	Outcome	
	Hardware	RegenOss and		Infection	Bone defect
BM	Circular external fixator	Vancomycin + Autologous bone	44	No recurrence	Bone healing
MS	None	Vancomycin	40	No recurrence	Bone healing
AG	Circular external fixator	Vancomycin	40	No recurrence	Bone healing
PR	None	Vancomycin	40	No recurrence	Bone healing
PG	Revision total hip arthroplasty	Vancomycin	40	No recurrence	Bone healing
DBS	Revision total hip arthroplasty	Vancomycin + Homologous bone	40	No recurrence	Bone healing
PE	Circular external fixator	Vancomycin	38	No recurrence	Bone healing
GG	Revision total hip arthroplasty	Vancomycin + Homologous bone	34	No recurrence	Bone healing
ZM	Circular external fixator	Vancomycin + Meropenem + Homologous bone	30	No recurrence	**Incomplente bone healing**
CM	Revision total hip arthroplasty	Vancomycin + Meropenem	26	No recurrence	Bone healing
PT	Osteosynthesis	Vancomycin + Meropenem	26	**Recurrence**	**No bone healing**
CMT	Circular external fixator	Vancomycin + Meropenem	26	No recurrence	**Incomplente bone healing**
SB	None	Vancomycin + Meropenem	24	No recurrence	Bone healing

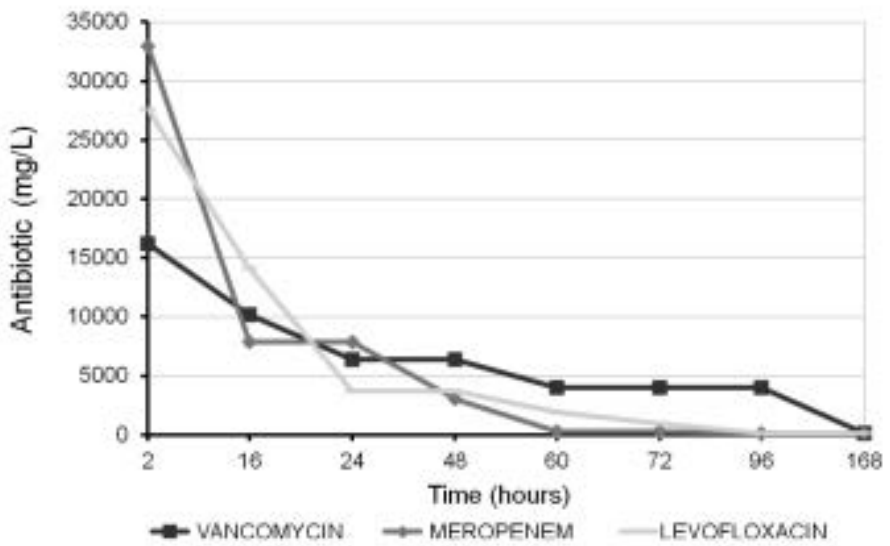
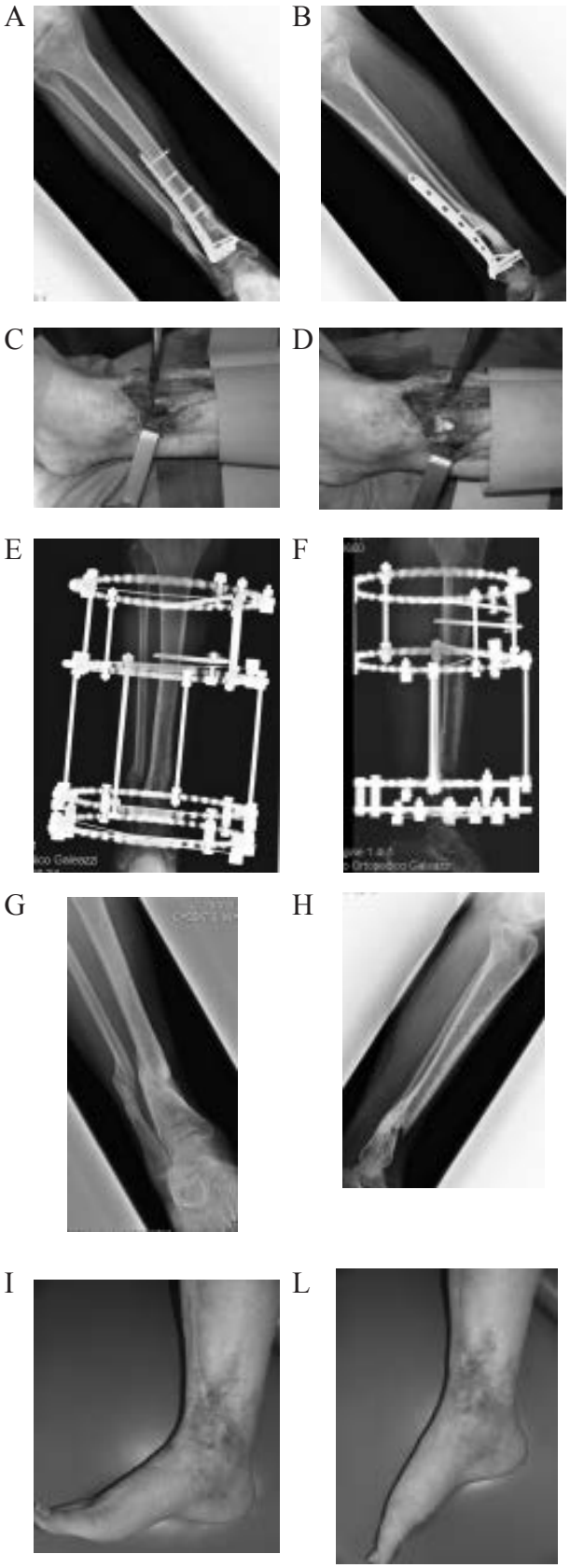


Fig. 1. *In vitro antibiotic release from RegenOss strips, tested by a microbiological assay. All antibiotics are completely released within 7 days. Each point represents the arithmetic average of three samples.*



RESULTS

In vitro release of antibiotics

Release of antibiotics from impregnated RegenOss was determined after 2, 16, 24, 48, 60, 96 and 168 h from loading. The antibiotics loaded on the product were fully recovered after one week, without significant differences between vancomycin, levofloxacin and meropenem, although some differences were observed in release kinetic. Overall, vancomycin release was slower than those of meropenem and levofloxacin. In fact, as reported in Fig. 1, more than 60% of meropenem or levofloxacin was recovered after 2 h of incubation, while only 30% of vancomycin was eluted at the same time. In line with this finding, at 48 h 98.5% of meropenem, 94.1% of levofloxacin and 76.3% of vancomycin were recovered in the medium.

Clinical results

At a minimum 24 months follow up (max 44, mean 34 months), no adverse events associated with the use of antibiotic-loaded RegenOss, either when used alone or in combination with bone grafts, such as local or systemic allergic reactions or rejection of the implant, were noted.

No recurrence of infection was observed in 12/13 (92.3%) patients; satisfactory bone healing with complete filling of the bone defect was observed in 10/13 cases (76.9%) (Fig. 2, 3), while unplanned further surgery was needed in 2/13 patients (15.3%).

One patient (PT) affected by severe rheumatoid arthritis with left hip prosthesis and sequelae of

Fig. 2. *AG, 51 years old female, first diagnosed with a closed, displaced, fracture of the distal third of her right tibia and fibula shaft after an high energy trauma. Treated with plate osteosynthesis, she developed a post-surgical infection and incomplete union with positive cultures to multi-resistant Staphilococcus aureus with metal exposure.* ***A, B****): Pre-operative radiographic finding 9 months after trauma;* ***C****): Intra-operative pictures showing the large bone defect after debridement;* ***D****): filled with vancomycin-loaded RegenOss;* ***E, F****): Post-operative x-ray, showing the external circular fixator in place;* ***G, H****): Radiographic control, two years after circular frame removal and three years after trauma;* ***I, L****): Complete bone and soft tissue healing, with satisfactory ankle range of motion.*

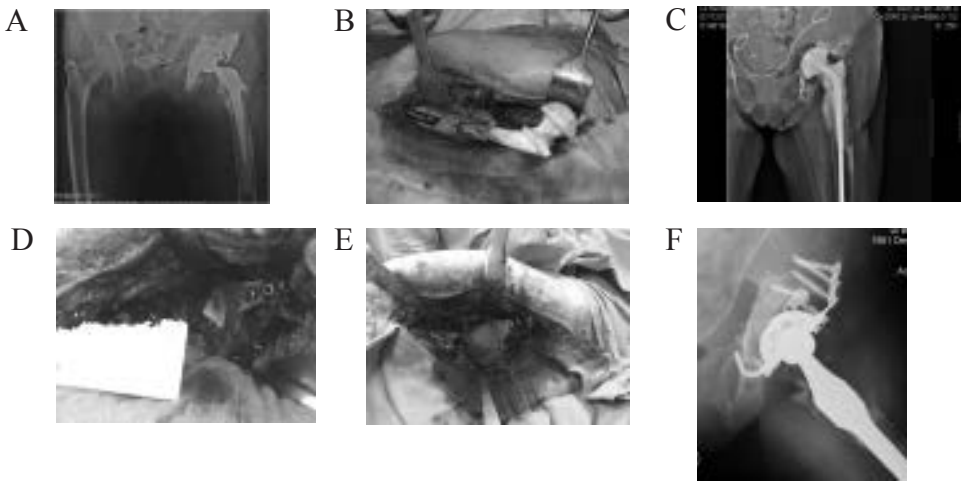


Fig. 3. *DBS, 52 years old caucasian female, peri-prosthetic infection in a cemented total hip arthroplasty, with extensive bone loss and after several previous surgeries and a previous renal transplant. Positive cultural examination (MRSA) and draining fistula.* ***A****): Pre-operative x-ray;* ***B****): Intra-operative picture at the time of infected implant removal and long stem spacer application;* ***C****): Post-operative x-ray;* ***D, E****): After 75 days the patients underwent spacer removal and bone stock reconstruction using antibiotic-loaded RegenOss and homologous morcellized bone graft to overcome large acetabular bone defect;* ***G****): Three years after surgery, radiographic findings show bone remodelling and bone stock reconstruction while the patient is infection free and able to walk long-distances with a cane.*

several revision and plastic surgeries for peri-prosthetic infection of her left knee, suffered a displaced femoral fracture at the tip of the hip and knee prostheses and that was treated with plate osteosynthesis and antibiotic-loaded RegenOss. However, early loosening of the synthesis, due to poor bone stock and quality and early recurrence of infection at the knee, finally lead to above-knee amputation. Another patient, affected by severe rheumatoid arthritis and several previous revision hip surgeries, suffered a loosening of the cup two-years after the two-stage procedure in which antibiotic-loaded RegenOss had been applied. This patient was successfully treated with further cup revision. Two more patients affected by septic non-union of the ankle after previous failed surgeries achieved incomplete bone healing and actually required a crutch and an orthopaedic shoe to walk, in the absence of infection recurrence.

DISCUSSION

This is the first study reporting on the ability of RegenOss, a collagen I and Mg-hydroxyapatite based bone substitute to effectively release different antibiotics *in vitro*. We also showed that the use of antibiotic-loaded RegenOss, alone or in combination with bone grafts, has not detrimental effect on bone healing or unwanted local or systemic side effects and may be helpful in controlling infection.

Until now autograft bone with its osteoinductive potential has been the material of choice when bone graft is needed, but in the presence of infection it is considered contraindicated (12, 20).

Many different methods have been used to treat the bone defect and the infection including bone transport through an external fixator, surgical debridement and bone defect filling with antibiotic-impregnated polymethylmethacrylate or antibiotic-loaded bone grafts or bone substitutes, but clinical resolution still remains a challenge, while persistent serum wound leakage using some calcium-based bone substitutes is a rather frequent occurrence (12, 20, 21).

Biomimetic materials, like RegenOss, are defined as materials able to promote bone formation, even if not proven able to induce heterotopic bone (22-27). Although shown to be highly biocompatible and effective to promote bone healing, evidence concerning their ability to work as local carrier of

antibiotic was lacking. This study points out that different antibiotics can be safely and easily loaded at surgery to RegenOss strips or patches based on available antibiograms and surgeon choice. Elution profile allows achieving satisfactory local levels of antibiotic in the first days after surgery, when the risk of bacterial adherence and biofilm formation on tissues and implants is maximum (2-4).

Clinical application, in this first continuous series of challenging patients, has shown the safety of the combination of RegenOss with vancomycin or with vancomycin and meropenem, with rate of infection control that, although in a very limited number of patients, compares well with other existing methods of local antibiotic delivery and even with antibacterial bioglass (21).

Moreover, the possibility to mix this medical device with bone grafts and its tolerability even in the presence of implanted biomaterials may be attractive for bone defect filling in revision surgery for failed joint prosthesis in infection or in high-risk patients.

This study has some limitations that should be considered before generalizing the results. *In vitro* antibiotic release from RegenOss has been investigated only for selected compounds; further studies are needed to evaluate the elution profile of other antibacterials and the bactericidial activity against other difficult-to-treat microorganisms, as for example Gram negative and multi-resistant strains. Moreover, we have no data concerning the ability of antibiotic-loaded RegenOss to resist biofilm formation or regarding possible induction of resistance.

The clinical series reported here is also limited in number and there is no control series. Larger, prospective comparative studies are recommended to confirm the promising results obtained in this preliminary study.

ACKNOWLEDGEMENTS

The study was partially funded by Finceramica SpA and the Italian Ministry of Health (project number 4062/2010).

REFERENCES

1. Romanò CL, Romanò D, Logoluso N, Drago L. Bone and joint infections in adults: a comprehensive classification proposal. Eur Orthop Traumatol 2011; 1(6):207-17.
2. Gristina AG, Costerton JW. Bacterial adherence and the glycocalyx and their role in musculoskeletal infection. Orthop Clin North Am 1984; 15(3):517-35.
3. Gristina AG, Shibata Y, Giridhar G, Kreger A, Myrvik QN. The glycocalyx, biofilm, microbes, and resistant infection. Semin Arthroplast. 1994; 5(4):160-70.
4. Gristina AG. Biomaterial-centered infection: microbial adhesion versus tissue integration. Science 1987; 237(4822):1588–95.
5. Schlickewei CW, Yarar S, Rueger JM. Eluting antibiotic bone graft substitutes for the treatment of osteomyelitis in long bones. A review: evidence for their use? Orthop Res Rev 2014; 71.
6. Levine SE, Esterhai JL, Heppenstall RB, Calhoun J, Mader JT. Diagnoses and staging. Osteomyelitis and prosthetic joint infections. Clin Orthop.1993; 295:77-86.
7. Thanni LOA, Osinupebi OA, Deji-Agboola M. Prevalence of bacterial pathogens in infected wounds in a tertiary hospital, 1995-2001: any change in trend? J Natl Med Assoc 2003 ;95(12):1189-95.
8. Lautenbach E, Patel JB, Bilker WB, Edelstein PH, Fishman NO. Extended-spectrum beta-lactamase-producing Escherichia coli and Klebsiella pneumoniae: risk factors for infection and impact of resistance on outcomes. Clin Infect Dis Off Publ Infect Dis Soc Am 2001; 32(8):1162-71.
9. Hake ME, Young H, Hak DJ, Stahel PF, Hammerberg EM, Mauffrey C. Local antibiotic therapy strategies in orthopaedic trauma: Practical tips and tricks and review of the literature. Injury 2015.
10. Kurien T, Pearson RG, Scammell BE. Bone graft substitutes currently available in orthopaedic practice: the evidence for their use. Bone Jt J 2013; 95B(5):583-97.
11. Barbanti Brodanò G et al Hydroxyapatite-Based Biomaterials *Versus* Autologous Bone Graft in Spinal Fusion. An *In Vivo* Animal Study. Spine 2014; 39:(11)661-8.
12. Geurts J, Chris Arts JJ, Walenkamp GHIM. Bone graft substitutes in active or suspected infection. Contra-indicated or not? Injury 2011; 42(2):S82-6.

13. Cormack AN, Tilocca A. Structure and biological activity of glasses and ceramics. Philos Trans R Soc Lond Math Phys Eng Sci 2012; 370(1963):1271-80.
14. Campana V, Milano G, Pagano E, et al. Bone substitutes in orthopaedic surgery: from basic science to clinical practice. J Mater Sci Mater Med 2014; 25(10):2445-61.
15. Hanssen AD. Local antibiotic delivery vehicles in the treatment of musculoskeletal infection. Clin Orthop 2005; 437:91-6.
16. Zalavras CG, Patzakis MJ, Holtom P. Local antibiotic therapy in the treatment of open fractures and osteomyelitis. Clin Orthop 2004; (427):86-93.
17. Romanò CL, Logoluso N, Meani E, et al. A comparative study of the use of bioactive glass S53P4 and antibiotic-loaded calcium-based bone substitutes in the treatment of chronic osteomyelitis: a retrospective comparative study. Bone Joint J 2014; 96(6):845-50.
18. Anagnostakos K, Schroder K. Antibiotic-Impregnated Bone Grafts in Orthopaedic and Trauma Surgery: A Systematic Review of the Literature. Int J Biomater 2012; e538061.
19. Beardmore AA, Brooks DE, Wenke JC, Thomas DB. Effectiveness of local antibiotic delivery with an osteoinductive and osteoconductive bone-graft substitute. J Bone Joint Surg Am 2005; 87(1):107-12.
20. Kanellakopoulou K, Giamarellos-Bourboulis EJ. Carrier systems for the local delivery of antibiotics in bone infections. Drugs 2000; 59(6):1223-32.
21. Romanò CL, Logoluso N, Meani E, Romanò D, De Vecchi E, Vassena C, Drago L. A comparative study of the use of bioactive glass BAG-S53P4 and antibiotic-loaded calcium based bone substitutes in the treatment of chronic osteomyelitis. Bone Joint J 2014; 96-B:845-50.
22. Tampieri A, Sandri M, Landi E, et al. Design of graded biomimetic osteochondral composite scaffolds. Biomaterials 2008; 29(26):3539-46.
23. Barbanera A, Longo GP, Vitali M, Sprio S, Tampieri A. Potential applications of synthetic bioceramic bone graft substitute in spinal surgery. Progress in Neuroscience 2013; 97-104.
24. Yamasaki Y, Yoshida Y, Okazaki M, et al. Action of FGMgCO3Ap-collagen composite in promoting bone formation. Biomaterials 2003; 24(27):4913-20.
25. Gruber HE, Rude RK, Wei L, Frausto A, Mills BG, Norton HJ. Magnesium deficiency: effect on bone mineral density in the mouse appendicular skeleton. BMC Musculoskelet Disord 2003; 4:7.
26. Bertinetti L, Drouet C, Combes C, et al. Surface Characteristics of Nanocrystalline Apatites: Effect of Mg Surface Enrichment on Morphology, Surface Hydration Species, and Cationic Environments. Langmuir 2009; 25(10):5647-54.
27. Crespi R, Capparè P, Gherlone E. Magnesium-enriched hydroxyapatite compared to calcium sulfate in the healing of human extraction sockets: radiographic and histomorphometric evaluation at 3 months. J Periodontol 2009; 80(2):210-8.

OSTEOARTICULAR ALLOGRAFTS IN PAEDIATRIC BONE TUMOR RECONSTRUCTION OF THE KNEE

D.A. CAMPANACCI[1], S. DURSKY[2], F. TOTTI[3], F. FRENOS[3], G. SCOCCIANTI[3], G. BELTRAMI[3] and R. CAPANNA[3]

[1]Department of Traumatology and General Orthopaedics, Azienda Ospedaliera Universitaria Careggi, Firenze, Italy; [2]Department of Orthopaedics and Traumatology, Kuala Lumpur General Hospital, Malaysia; [3]Department of Orthopaedic Oncology and Reconstructive Surgery, Azienda Ospedaliera Universitaria Careggi, Firenze, Italy

Osteoarticular allografts represent a reconstructive option after bone tumor resection around the knee in growing children. The major advantage is the chance to preserve the growth plate of the remaining bone, but the disadvantage is the high failure rate eventually requiring definitive prosthetic replacement at skeletal maturity. We retrospectively reviewed 22 patients who underwent osteoarticular allograft reconstructions of the distal femur (16) or proximal tibia (6). There were 12 females and 10 males with an average age at surgery of 11 years (7-15). The diagnosis was osteosarcoma in 19 cases and Ewing sarcoma in 3. All patients underwent pre- and post-operative chemotherapy. At an average follow-up of 103 months (12-167), 18 patients (82%) were alive and 4 had died (18%). We observed 10 allograft failures requiring prosthetic replacement, 6 in distal femur and 4 in proximal tibia reconstructions. At last follow-up 8 allografts (36%) were still in place. Overall allograft survival was 79.6% at five and 45.8% at ten years. In distal femur, allograft survival was 86.2% at five and 59.1% at ten years. In proximal tibia, allograft survival was 62.5% at 5 years and 31.2% at 67 months. Average limb shortening was 3 cm (0-5) in 8 patients with the allograft still in situ and 2 cm (0-4) in 10 patients after prosthetic replacement. Average MSTS functional score of the whole series was 25 (83.7%). The MSTS score of patients after revision with prosthetic replacement was 24 (80%) while patients who still had the allograft retained had an average MSTS scores of 26.8 (89.3%). In conclusion, osteoarticular allograft reconstruction of the knee after bone tumor resection in pediatric age can be considered a temporary solution with the aim to limit limb length discrepancy before definitive prosthetic replacement after skeletal maturity.

In orthopaedic oncology, bridging the massive bone defect after resection has always been an evolving process since the concept of limb salvage surgery was made possible in the early 1980's (1-7).

The reconstruction is more challenging in paediatric age group where primary malignant bone tumours are most often encountered (8-9). The growth plates of the distal femur and proximal tibia determine the definitive length of the lower limbs, and tumour resection involving the physis will cause limb length discrepancy at skeletal maturity. However, limb length discrepancy is only the final hurdle if issues of the ideal construct and materials used to bridge the massive bone gap are overcome during the active medical and surgical period.

Reconstructive options of the knee that are

Key words: osteoarticular allograft, knee, bone tumor

Mailing address: Domenico Andrea Campanacci MD, Department of Traumatology and General Orthopaedics, Azienda Ospedaliera Universitaria Careggi, Largo Brambilla 3, 50134 Firenze, Italy
Tel.: +39 055 7946374
Fax: +39 055 7948253
e-mail: campanaccid@gmail.com

0393-974X (2015)

available for limb salvage in the skeletally immature patient have included allografts, expandable megaprosthesis and allograft-prosthetic composites (3, 4, 10-15).

In the growing child, limb salvage procedures that rely on endoprosthesis or bone allografts present unique challenges. These include: a) the small size of the paediatric skeleton with fitting concerns for the implants. Last generation non-invasive lengthening prostheses require extensive minimal resection length and the magnetic gear is larger than previous models. Osteoarticular allografts are retrieved from adult donors and dimensional matching is difficult or impossible in paediatric patients; b) the growth potential of the patient and the expected final length of the unaffected limb, with the need for correction of the ensuing limb-length discrepancy; c) the concerns of prosthetic stem fixation in immature bone with the risk of periprosthetic cortical stress-shielding; d) the need for durable reconstruction in this young population.

Osteoarticular allograft (OA) reconstruction of the knee offers the chance to restore a mobile joint preserving the healthy bone stock as much as possible and at the same time sparing one of the growth plates of the knee. The use of OA on the knee in growing children provides the following advantages: the resection length can be decided on the basis of tumour extension and no minimal resection length is required; except for plate fixation, the medullary canal of healthy bone remains unviolated and no cortical stress-shielding is expected; the growth plate opposite to the resection site remains intact resulting in decreased final limb length discrepancy.

In literature, a high mechanical complication rate was observed after OA reconstruction around the knee, eventually leading to surgical revision with implant removal and definitive prosthetic replacement (16-19). In paediatric patients, OA allografts of the knee can be considered a temporary reconstructive solution to maintain bone stock and to decrease final limb length discrepancy, before definitive prosthetic replacement after skeletal maturity.

We reviewed our paediatric patients treated with a distal femur and proximal tibia osteoarticular bone allograft replacement after resection of primary bone sarcoma with the aim to evaluate the long-term oncologic and functional outcome.

In particular, we tried to answer the following questions: 1) What was the functional outcome of the reconstruction; 2) What was the functional outcome and final limb length discrepancy at skeletal maturity after definitive revision; 3) Can we consider OA of the knee as safe biologic temporary spacer effective in maintaining bone stock until definitive prosthetic replacement at skeletal maturity?

MATERIALS AND METHODS

We retrospectively reviewed 22 paediatric patients who underwent osteoarticular bone allograft reconstruction after resection of distal femur or proximal tibia bone sarcoma between 1997 and 2008. A summary of the demographics, clinical data, complications and case outcomes is shown in Table I.

In this study group there were 12 girls and 10 boys with an average age at time of surgery of 11 years (7 to 15) and a mean follow-up duration of 103 months (12 to 167).

The histologic diagnosis was osteosarcoma in 19 cases (15 distal femur, 4 proximal tibia) and Ewing sarcoma in 3 cases (1 distal femur, 2 proximal tibia). Osteoarticular allograft reconstruction included the distal femur in 16 cases and the proximal tibia in 6 cases. All patients underwent neoadjuvant and adjuvant chemotherapy based on respective osteosarcoma and Ewing sarcoma national protocol.

All the patients received perioperative antibiotics according to the institution's protocol. Medial or lateral surgical approach was performed with excision of the previous biopsy scar. An intra-articular resection was performed in all patients. The level of bone resection was determined preoperatively by the post neoadjuvant chemotherapy MRI images obtained prior to surgery. A sample from the host medullary canal at osteotomy site was taken for frozen section before proceeding with reconstruction. One physis of the knee was preserved in all patients. The overall average length of bone resection was 15.6 cm (11 to 20).

The final histopathology report of the resection specimen revealed 20 out of 22 patients had wide resection margins and 2 had marginal margins. However, the 2 patients reported to have marginal resection did not have any local recurrence and are alive and disease free at the time of writing.

The Institution's in-house bone bank supplied the required non-irradiated fresh deep frozen massive allografts. Allografts were harvested under sterile conditions and stored frozen at -80°C. After tumour resection, the pre-matched allografts were taken out

Table I. *Osteoarticular allografts in 22 patients: demographics, clinical data, complications and oncological outcomes.*

Case No.	FU (mth)	Age Operated	Current Age	Sex	Site	Resection (cm)	Cement	Meta-stasis	LLD (cm)	Infection (4/22; 18%)	Allograft Fracture (10/22; 45%)	Non-Union (3/22; 14%)	Allograft Outcome	Oncologic Outcome	MSTS Score
1	113	12	22	F	DF	20	Yes	No	3.5	No	No	No	Retained	CDF	[illegible]
2	98	[illegible]	[illegible]	M	DF	[illegible]	Yes	No	[illegible]	No	No	Yes	Retained	CDF	[illegible]
3	167	9	24	F	DF	17	No	No	[illegible]	No	Yes	No	Replaced	CDF	[illegible]
4 (D)	61	8	(D)	F	DF	[illegible]	Yes	Yes	2	No	No	No	Retained	DOD	29
5	107	[illegible]	[illegible]	F	DF	16	Yes	No	0	No	No	No	Retained	CDF	[illegible]
6 (D)	97	10	(D)	F	DF	[illegible]	No	Yes	[illegible]	Yes	No	No	Retained	DOD	[illegible]
7	90	[illegible]	[illegible]	M	DF	17	Yes	No	5	No	No	No	Retained	CDF	28
8	116	12	22	M	DF	18	No	No	2	No	No	No	Retained	CDF	22
9	108	8	[illegible]	F	PT	[illegible]	Yes	No	Lengthening	No	Yes	No	Replaced	CDF	[illegible]
10	124	11	24	M	DF	12	Yes	No	3	No	Yes	No	Replaced	CDF	28
11	[illegible]	10	[illegible]	F	PT	[illegible]	No	Yes	[illegible]	Yes	Yes	No	Replaced	NED	[illegible]
12 (D)	47	8	(D)	F	DF	[illegible]	No	No	5	No	No	No	Retained	DOC	[illegible]
13 (D)	[illegible]	[illegible]	(D)	F	DF	[illegible]	Yes	[illegible]	[illegible]	No	No	No	Retained	DOD	[illegible]
14	65	10	18	M	DF	15	Yes	No	Lengthening	No	Yes	Yes	Replaced	CDF	[illegible]
15	56	8	[illegible]	F	DF	16	Yes	No	[illegible]	No	No	No	Retained	CDF	29
16	126	15	25	M	DF	18	Yes	No	0	No	Yes	No	Replaced	CDF	[illegible]
17	135	13	24	F	PT	13	Yes	No	1	Yes	Yes	Yes	Replaced	CDF	27
18	133	[illegible]	28	M	PT	16	Yes	No	[illegible]	No	Yes	No	Replaced	CDF	[illegible]
19	120	7	20	F	PT	16	Yes	No	0	No	Yes	No	Replaced	CDF	28
20	[illegible]	[illegible]	[illegible]	M	PT	[illegible]	Yes	No	[illegible]	No	Yes	No	Replaced	CDF	[illegible]
21	64	13	18	M	PT	11	Yes	No	3	Yes	No	No	Retained	CDF	28
22	[illegible]	10	[illegible]	M	PT	[illegible]	No	No	[illegible]	No	No	No	Retained	CDF	[illegible]

Legend: FU = Follow-up in months; [illegible]; (D) Deceased; Sex: F (Female), M (Male); DF = Distal Femur; PT = Proximal Tibia; DOD = Died of Disease; DOC = Died of Other Cause

of their packaging and thawed in a warm normal saline solution mixed with Rifampicin (15). An anatomically matched size allograft was used whenever possible. While 8 patients had good anatomical matching, 14 had oversized allograft as bone donors were adults. The allografts were trimmed to the proper length and soft tissue structures were prepared for implantation. Osteosynthesis was performed with long 4.5mm plates and screws that spanned the total length of the allograft and extended to the femoral or tibial diaphysis. A compression osteosysnthesis of the host-allograft osteotomy was performed in order to achieve a rigid fixation. The knee joint was reconstructed with unresorbable sutures beginning with the posterior cruciate ligament and following in order with the posterior capsule, the anterior cruciate ligament and the lateral and medial collateral ligaments. The objective was to obtain stability avoiding excessive posterior stiffness with consequent risk of extension lag.

The extensor mechanism continuity was maintained in distal femur resections while, in proximal tibia reconstructions, the patellar tendon of the patient was sutured to the patellar tendon of the allograft with unresorbable sutures, taking care to restore a correct isometry and full flexion range of motion.

Intramedullary cement was used in 16 cases while 6 allografts were implanted without cement. In proximal tibia resection, a medial gastrocnemius flap was used to cover the anteromedial surface of the allograft as a primary procedure in 4 cases and as salvage procedure after superficial wound dehiscence in 2 cases.

Postoperatively, the operated limb was immobilized in a splint, which was changed to a brace at drains removal after 48 h. The brace was removed after one month and passive and active knee motion was encouraged. Ambulation was initiated without weight bearing of the affected extremity and gradual progression to partial and full weight bearing was allowed, depending on radiographic evidence of healing. The follow up of patients was managed in an integrated out-patient clinic where they were seen by the surgeon, the oncologist and physiotherapist in one setting. The patients were seen every three months during the first two years, followed by every four months in the third year and 6 months up to five years from surgery, then yearly.

At the latest follow-up, a radiographic panoramic view of the lower limbs was performed to examine limb-length discrepancy and to assess physeal growth. Functional outcome was assessed using MSTS scoring system.

Postoperative complications recorded were infection, non-union, fracture and articular degeneration. Complication treatment and outcome were analysed.

Allograft's survival, with allograft removal and prosthetic replacement as end point, was assessed using the 'survfit' function from the Kaplan-Meier 'survival' package in R, an open source application for statistical computation (20, 21).

RESULTS

At an average follow up of 103 months (12-167), 18 patients (82%) were alive and 4 had died (18%). Seventeen patients were continuously disease free (77%) and one was alive with no evidence of disease. Three patients died of disease and 1 died of another cause (leukaemia).

The patient who was alive with no evidence of disease presented a local recurrence 8 months after primary surgery which was treated with surgical excision with wide margins. After 17 months from primary surgery, pulmonary metastasis was detected. Open surgical metastatectomy was done followed by chemotherapy treatment. At last follow up, after 142 months from primary surgery, this patient was alive with no evidence of disease.

Distal femur

Sixteen patients with an average age of 11 years (8 to 15) underwent osteoarticular allograft reconstruction of the distal femur. Four patients died at 1, 5, 6 and 8 years from primary surgery. Average length of resection for distal femur reconstruction was 16.1 cm (12 to 20). There were 2 infections in the distal femur series, both healed after surgical debridement. One patient developed infection after 10 months from primary allograft reconstruction. Complete healing of the infection was observed after surgical debridement and the complication did not compromise the osteotomy union.

The second patient presented a post-operative wound dehiscence after revision surgery with a composite prosthesis (preserving the allograft) for painful joint degeneration after 7 seven years from primary surgery. Out of twelve surviving patients, ten achieved primary union of the osteotomy with fusion time ranging between 10 to 17 months (mean 13 months). One patient presented a delayed union with resorption of the medial side of the allograft observed at 24 months. This complication was treated conservatively and eventually satisfactory union was observed at follow up. One patient developed a non-union with hardware failure at 24 months. Implant revision with screws replacement and autologous iliac crest bone grafting was performed. Subsequently progressive union of the osteotomy was observed. Allograft fracture occurred in 6 patients. The average time to fracture was 91 months (35 to 142).

All patients underwent surgical revision with allograft removal and modular prosthesis

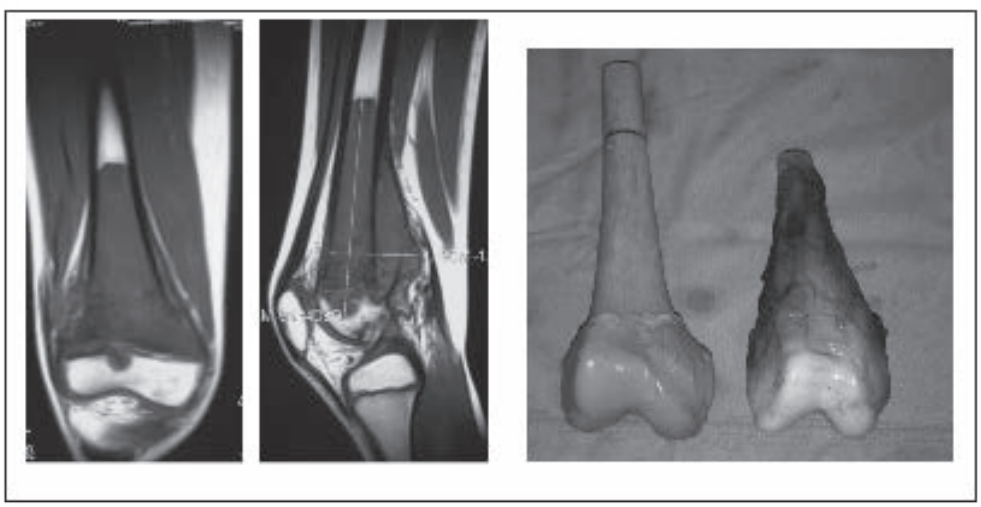


FIG. 2. *Distal femur Osteosarcoma in an 8 years old girl.* ***A, B****): Coronal and sagittal view of pre-operative MRI showing tumour extension through the growth plate into the epiphysis;* ***C****): Anatomical matching between distal femur allograft and resected specimen of the distal femur.*

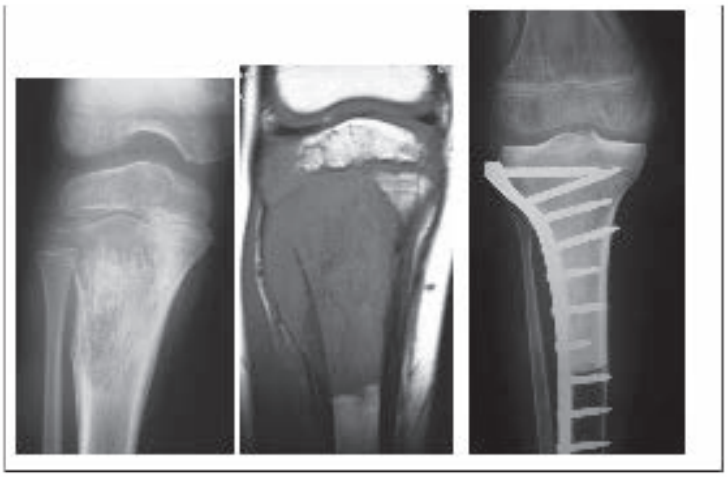


FIG. 3. *Proximal tibia Ewing sarcoma in a 10 years old boy.* ***A****): A-P radiographic view of the tumour before neoadjuvant chemotherapy;* ***B****): Coronal view of preoperative MRI showing epiphyseal involvement;* ***C****): Postoperative radiograph showing osteoarticular allograft reconstruction with periarticular plate osteosynthesis.*

reconstruction in 4 cases and lengthening prosthesis in two cases. The lengthening prosthesis was used in two patients with shortening of 6 cm in one case and 6.5 cm in one case. A non-invasive expandable prosthesis was used and in both patients the lengthening process was still ongoing at time of writing. Epiphysiodesis of the contralateral knee was performed in 2 cases. In these two cases, the final length discrepancy at skeletal maturity of lower limbs was 3 cm and 4 cm respectively. At the time of the last review of 12 surviving patients, 11 had achieved skeletal maturity. In four patients the allograft had been replaced by modular prosthesis and in two patients by expandable prosthesis.

Average limb length discrepancy after modular prosthesis revision was 2.3 cm (0 to 4) while in patients with expandable prosthesis the lengthening process was still ongoing at time of writing. Six patients still had the allograft in situ and average limb length discrepancy was 3.1cm (0 to 5).

Proximal tibia

Six patients, 4 males and 2 females with an average age of 12 years (7 to 14), underwent proximal tibia reconstruction. One patient was lost at follow-up since he went back home abroad. The average length of resection was 14.6 cm (9 to 17). All 6 patients were alive and continuously disease free at the last follow up. Two patients presented a wound dehiscence with superficial infection within 2 months from allograft implantation. Both cases required a surgical debridement followed by rotational flap coverage of medial gastrocnemius and skin graft. After revision, the wound successfully healed in both cases. One non-union of the osteotomy was observed at 24 months which was successfully treated with autologous bone graft augmentation. The average time to union of the osteotomy site was 11.5 months (9 to 14). Four patients presented an allograft fracture, two patients after 5 years and two after 6 years from primary surgery. All 4 patients underwent surgical revision with allograft removal and modular prosthesis reconstruction in 3 cases and lengthening prosthesis in 1 case. The non-invasive lengthening prosthesis was implanted in a patient who underwent primary surgery when she was 7 years old and eventually developed 5.5 cm shortening at 14 years of age. The fractured allograft was replaced with a non-invasive expandable prosthesis, which was converted to a definitive implant after lengthening. One patient, currently after 5 years from primary surgery, is under evaluation for painless articular degenerative changes. All 6 patients achieved skeletal maturity and, at final follow-up, limb length discrepancy ranged from 0 to 3 cm (average 2 cm over 5 evaluable patients).

Fifteen patients were available for MSTS functional evaluation. The average MSTS score for the whole series was 25.1 (83.7%). The MSTS score of patients after revision with prosthetic replacement was 24 (80.0%) while patients who still had the allograft retained had an average MSTS scores of 26.8 (89.3%).

The overall allograft survival was 79.6% at 5 years and 45.8% at 10 years. Distal femur allografts showed a longer survival than proximal tibia allografts (86.2% *vs* 62.5% at 5 years and 59.1% at 10 years *vs* 31.2% at 67 months respectively) ($P<0.0065$) and these data are in line with results from previous publications. Fig. 4 and 5 show the Kaplan-Meier graphs. A comparison of results from similar studies is shown in Table II.

DISCUSSION

The knee joint (distal femur and proximal tibia) is the most frequent site of occurrence of primary bone tumours in children. When there is no tumour extension inside the joint space and an intra-articular resection can be performed with safe surgical margins, osteoarticular massive allograft can be used for reconstruction. Other reconstructive solutions in paediatric age include rotationplasty, arthrodesis, lengthening prosthesis and allograft-prosthesis composites. Except rotationplasty, which can be considered a definitive solution, all techniques used for knee joint reconstruction in growing children represent the first step of a limb-salvage project which will be followed by other surgical or non-surgical corrective steps.

Last generation expandable prosthesis are very attractive for the non-invasive electromagnetic device allowing lengthening in outpatient setting. However, the device dimension can be a problem in younger children and the minimum resection length required may lead to unnecessary loss of bone stock. Osteoarticular allograft of the knee have the advantage to preserve patient's healthy bone stock and to preserve the integrity of adjacent growth plate in paediatric patients. Due to the high rate of mechanical complication reported with osteoarticular allograft reconstruction around the knee, currently allograft-prosthesis composites or modular prosthesis are preferred for knee reconstructions in adults. On the other hand, in paediatric age osteoarticular allografts of the knee are still considered a valuable reconstructive option. The reconstruction provide an excellent function during the time of growth, a limitation of limb shortening (preserving the integrity of adjacent growth plate) and, in case of failure, it can be definitively replaced at skeletal maturity with a modular prosthesis. During growth, limb length discrepancy may be furtherly limited by contralateral epiphysiodesis, and in case of very young children with consistent final shortening, a lengthening prosthesis may be implanted at skeletal maturity.

This study has limitations. One limitation is the inclusion of both distal femur and proximal tibia in the same series, but we decided to analyse both sites to allow a comparison of the outcome of different reconstructions. Another limitation is the limited follow up (average 103 months) but all patients except one had reached skeletal maturity at latest control and the data allowed an evaluation of the functional outcome and limb length discrepancy at the end of growth.

Excellent oncologic results were achieved in the present series of patients, all affected by high grade primary malignant bone tumours. Local recurrence was observed in 4.5% of cases and 77% of patients were continuously disease free at final follow up. The overall functional results of evaluable patients at final follow up after skeletal maturity showed an average MSTS score of 84%. Functional score was higher in patients who were still having the allograft in situ as compared to the patients who had the allograft replaced by a prosthesis. Following the revision with allograft replacement by an endoprosthesis, the major functional improvement achieved is joint stability. However, the surgical trauma of the procedure, especially if associated to an intraoperative limited lengthening (1-2 cm), may lead to a decrease of range of motion if compared to the preoperative status. Moreover, it is common that osteoarticular allografts of the knee provide an excellent function to the patient until a major complication leads to a failure requiring surgical revision.

A non-union of the osteotomy was observed in 14% of cases. Other studies have reported a non-union rate of the allografts ranging from 9% to 20%

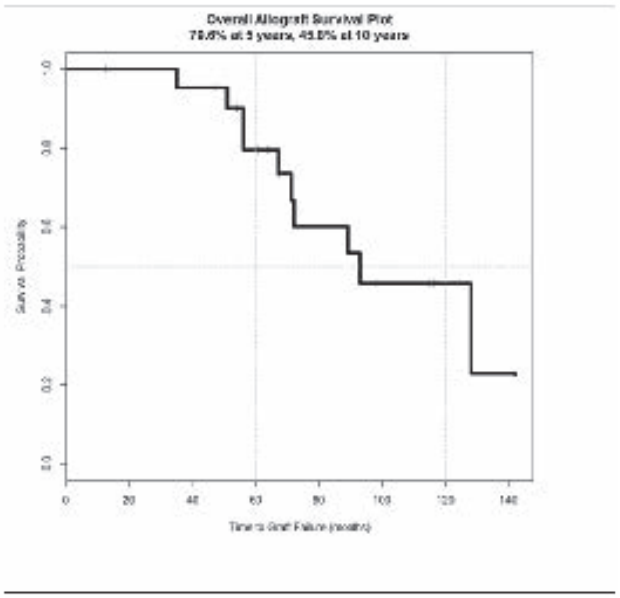


Fig. 4. *Overall osteoarticular allograft survival considering prosthetic revision as end point.*

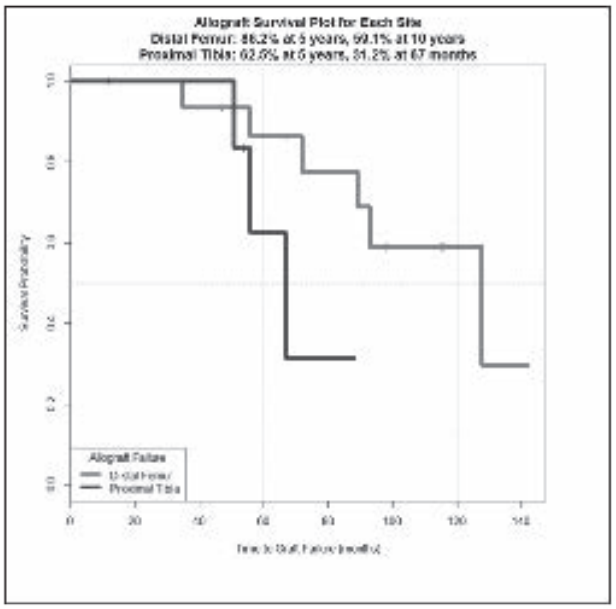


Fig. 5. *Survival for distal femur and proximal tibia osteoarticular allograft considering prosthetic revision as end point.*

(8, 12, 16, 17). Union of the allografts depends on the host bone osteoinductive ability to repair the gap, a process that can be substantially delayed by chemotherapy (3). All patients in this series received both neoadjuvant and adjuvant chemotherapy. The fixation stability and the adequate contact between the allograft and host bone are also affecting the rate of union. In all patients of this series a transverse osteotomy was performed and a compression plate was used for fixation. One non-union healed spontaneously with time, while in 2 cases union was achieved after screw replacement and autologous cancellous bone graft apposition.

Mechanical failure is a frequent complication of massive allograft reconstructions and in osteoarticular allografts most frequently the fracture involves the articular surface. Progressive degenerative changes of the joint space are usually observed eventually leading to a subchondral collapse (18). In literature, a fracture rate ranging from 9% to 45% was reported in osteoarticular allografts. All 22 patients in this series had the same mode of fixation, a long compression plate spanning the entire length of the allograft. Intramedullary cement was used to improve mechanical strength in 16 cases but no correlation was found between the use of cement and the incidence of allograft fracture. In distal femur reconstructions, 6 fractures of the allograft were observed, 1 at diaphysis and 5 at articular level. The distal femur fracture of the diaphysis occurred in one patient at 3 years after primary allografts fixation. Articular fractures of the distal femur occurred at an average of 7.5 years from primary surgery. In the proximal tibia series no diaphyseal fracture was observed. The 4 fractures that occurred in the proximal tibia group were all at the articular region. The average time to the documented radiographic collapsed of the tibia articular surface was 5.5 years. All these patients had the allograft removed and replaced with endoprosthesis. Another patient who still has the proximal tibia allograft retained has radiographic signs of articular surface degeneration and is currently under observation. According to other reports on osteoarticular allograft, articular fractures and failures requiring revision were more frequent in proximal tibia than in distal femur. Considering that in proximal tibia replacement both meniscus are resected and replaced with allogenic tissue, it can be postulated that retaining the native meniscus in distal femur replacements could delay the degenerative process of the articular cartilage of the allograft allowing a longer survival of the

reconstruction.

The overall infection rate for the whole series was 18%. However, we did not encounter any deep infection that warranted for allograft removal both in the distal femur and proximal tibia group. As expected, the infection rate in the proximal tibia (33.3%) was more than twice that of distal femur (12.5%). All infections healed after surgical debridement, requiring an additional rotational flap coverage in the 2 cases of tibia reconstruction. Other published studies have reported infection rates between 4.5% to 20% in massive allograft surgery (3, 16, 17, 19). According to the literature and on the basis of our results, primary medial gastrocnemius rotational flap coverage with or without skin graft is recommended in proximal tibia reconstructions.

At the time of writing, all but one of 18 surviving patients had attained skeletal maturity. Six distal femur and 4 proximal tibia patients had their allografts replaced with mega prosthesis (expandable prosthesis in 3 cases). Two patients were still in the lengthening process while the remaining eight patients had an average final limb length discrepancy of 2 cm, ranging from 0 to 4 cm. Eight patients still had the initial allografts construct in situ with an average limb length discrepancy of 3 cm, ranging from 0 to 5 cm.

In conclusion, osteoarticular massive allograft reconstruction of the knee after bone tumour resection in growing children was successful in providing an excellent functional result with a limited final limb shortening. Due to the high mechanical failure rate of the reconstruction, this procedure has to be considered a temporary solution until the age of skeletal maturity when the allograft can be eventually replaced by a definitive prosthetic implant.

REFERENCES

1. Allison DC, Carney SC, Ahlmann ER, Hendifar A, Chawla S, Fedenko A, Angeles C, Menendez LR. A meta-analysis of osteosarcoma outcomes in the modern medical era. Sarcoma 2012; 704872.
2. Huang TL, Chen TH, Chen WYK, Chen WM, Liu CL, Lo WH. Allograft arthrodesis of the knee in high-grade osteosarcoma. J Chin Med Assoc 2005; 68(9):425-30.
3. Muscolo DL, Ayerza MA, Aponte-Tinao L, Farfalli G. Allograft reconstruction after sarcoma resection in children younger than 10 years old. Clin Orthop Relat Res 2008; 466(8):1856-62.
4. Abed YY, Beltrami G, Campanacci DA, Innocenti M, Scoccianti G, Capanna R. Biological reconstruction after resection of bone tumours around the knee. Long-term follow-up. J Bone Joint Surg Br 2009; 91(10):1366-72.
5. Cannon MSR. Bone Tumors: Limb Salvage. In The Surgery of Childhood Tumors Springer Berlin Heidelberg 2008; 321-51.
6. San-Julian M, Vázquez-García BL, Sierrasesúmaga L. Limb Salvage in Children. Eur Surg Ortho and Traum 2014; 4251-80.
7. Bellanova L, Paul L, Docquier PL. Surgical guides (patient-specific instruments) for pediatric tibial bone sarcoma resection and allograft reconstruction. Sarcoma 2013; 2013:787653
8. Forriol F, San-Julian M, Cañadell J. Location of sarcomas within bone: the growth plate. In Pediatric Bone Sarcomas. Springer 2009; 57-70.
9. Ottaviani G, Jaffe N. The epidemiology of osteosarcoma. In Pediatric and Adolescent Osteosarcoma. Springer 2010; 3-13.
10. Raskin KA, Hornicek F. Allograft reconstruction in malignant bone tumors: indications and limits. In Treatment of Bone and Soft Tissue Sarcomas. Springer 2009; 51-58.
11. Holzer LA, Leithner A. Biological Extremity Reconstruction after Sarcoma Resection: past, present and future. Sarcoma 2013; 529349.
12. Toy PC, White JR, Scarborough MT, Enneking WF, Gibbs CP. Distal femoral osteoarticular allografts: long-term survival, but frequent complications. Clin Orthop Relat Res 2010; 468(11):2914-23.
13. Ogilvie CM, Crawford EA, Hosalkar HS, King JJ, Lackman RD. Long-term Results for Limb Salvage with Osteoarticular Allograft Reconstruction. Clin Orthop Relat Res 2009; 467:2685–90.
14. Hwang N, Grimer RJ, Carter SR, Tillman RM, Abudu A, Jeys LM. Early results of a non-invasive extendible prosthesis for limb-salvage surgery in children with bone tumors. J Bone Joint Surg Br 2012; 94(2):265-9.
15. Van Isacker T, Cornu O, Barbier O, Dufrane D, de Gheldere A, Delloye, C. Bone Autografting, Al-

lografting and Banking. European Surgical Orthopaedics and Traumatology: The EFORT Textbook. Springer 2014; 77-90.
16. Ogilvie CM, Crawford EA, Hosalkar HS, King JJ, Lackman RD. Long-term results for limb salvage with osteoarticular allograft reconstruction. Clin Orthop Relat Res 2009; 467(10):2685-90.
17. Campanacci L, Manfrini M, Colangeli M, Alì N, Mercuri M. Long-term results in children with massive bone osteoarticular allografts of the knee for high-grade osteosarcoma. J Ped Orthop 2010; 30(8):919-27.
18. Enneking WF, Campanacci DA. Retrieved human allografts: a clinicopathological study. J Bone Joint Surg Am 2001; 83(7):971-86.
19. Kohler R, Lorge F, Brunat-Mentigny M, Noyer D, Patricot L. Massive bone allografts in children. Intern Orthop 1990; 14(3):249-53.
20. Terry Therneau and original S-Plus to R port by Thomas Lumley (2011). Survival: Survival analysis. R package version 2.37-7. http://CRAN.R-project.org/package=survival.
21. R Development Core Team (2010). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.

DOES TOTAL KNEE REPLACEMENT MODIFY FLEXION AXIS OF THE KNEE ON FRONTAL AND AXIAL PLANE REGARDLESS FROM LIMB ALIGNMENT?

D. BRUNI[1], L. BRAGONZONI[2], M. BONTEMPI[3], F. IACONO[1], M.P.NERI[1], S. BIGNOZZI[4], S. ZAFFAGNINI[1] and M. MARCACCI[5,6]

[1]2nd Orthopaedics and Traumatologic Clinic, Rizzoli Orthopaedic Institute, , Bologna, Italy; [2]Biomedical and Neuromotor Sciences - DIBINEM - The Alma Mater Studiorum, University of Bologna, Italy; [3]Biomechanics Laboratory, Codivilla-Putti Research Center, Bologna, Italy; [4]Orthokey Italia Srl, Italy; [5]Director of the 2nd Orthopaedics and Traumatologic Clinic, Rizzoli Orthopaedic Institute, Bologna, Italy; [6]Director of Biomechanics and Technologic Innovation Laboratory and Nanobiotechnology Laboratory, Codivilla-Putti Research Center, Bologna University, Bologna, Italy

The optimal reference for rotational positioning of femoral component in total knee replacement (TKR) is debated. Navigation has been suggested for intra-op acquisition of patient's specific kinematics and functional flexion axis (FFA). The main purpose of the present study is to prospectively investigate whether pre-operative FFA in patients with osteoarthritis (OA) and varus alignment changes after TKR and whether a correlation exists between post-op FFA and pre-op alignment. A navigated TKR was performed in 108 patients using a specific software to acquire passive joint kinematics before and after TKR. The knee was cycled through three passive range of motions (PROM), from 0° to 120°. FFA was computed using the mean helical axis algorithm. The angle between FFA and surgical TEA was determined on frontal (α^f) and axial (α^a) plane. The pre- and post-op hip-knee-ankle angle (HKA) was determined. Post-op FFA was different from pre-op FFA only on frontal plane. No significant difference was found on axial plane. No correlation was found between HKA-pre and α^A-pre. A significant correlation was found between HKA-pre and α^F–pre. The study concluded that TKR modifies FFA only on frontal plane. No difference was found on axial plane. Pre-op FFA is in a more varus position respect to TEA. The position of FFA on frontal plane is dependent on limb alignment. The present study has demonstrated TKR modifies the position of FFA only on frontal plane. The position of FFA on axial plane is not dependent on the amount of varus deformity and is not influenced by TKR. Level of evidence, IV, case series.

Rotational alignment of the femoral component is fundamental to obtain a correct tibio-femoral (TF) and patello-femoral (PF) kinematics after total knee replacement (TKR) (1-4). Malrotation of the femoral component reduces implant survivorship and impairs functional results (1). Many reference systems and anatomical landmarks have been suggested to improve accuracy in the positioning of components but they demonstrated to have a low repeatability and a high intra- and inter-observer variability (3, 5-8). Therefore, the optimal surgical references to optimize rotational positioning of the femoral component during a TKR are still debated.

Navigation has been suggested to improve accuracy of implant positioning and to reduce the number of outliers in frontal and axial alignment (9-12). In any case, the accuracy in the acquisition of the anatomic landmarks used to plan implant

Key Words: TKA, functional flexion axis, knee kinematic

Mailing address: Danilo Bruni, MD,
2nd Orthopaedics and Traumatologic Clinic,
Rizzoli Orthopaedic Institute,
Via Pupilli 1, 40136, Bologna, Italy
Tel.:+39 051 6366506
Fax: +39 051 583789;
e-mail address: d.bruni@biomec.ior.it; drdanilobruni@gmail.com

0393-974X (2015)

positioning depends on the surgeon's experience (13-14). Furthermore, the use of navigation in TKR has failed to provide superior clinical results and implant survivorship compared to conventional procedures (15-16).

Despite these limitations, navigation has been recently used for intra-operative acquisition of the patient's specific kinematics and to describe the individual functional flexion axis (FFA) according to different mathematical algorithms (17). Its reliability in determining femoral component rotation in a TKR has been demonstrated in a cadaveric model (17) but not *in vivo*. Moreover, no data is reported in literature regarding FFA modification after a TKR compared to pre-operative condition, nor whether a correlation exist between pre-operative limb alignment and post-operative FFA.

The main purpose of the present study was to prospectively investigate whether pre-operative FFA of the knee in patients with end-stage osteoarthritis (OA) and varus alignment changes after TKR. The secondary purpose was to determine whether a correlation exists between post-operative FFA and the amount of pre-operative varus deformity.

MATERIALS AND METHODS

A unilateral cruciate-retaining (CR), mobile-bearing (MB) TKR (Gemini-Light, Waldemar Link, Hamburg, Germany) was performed in a series of 108 consecutive patients with primary knee osteoarthritis (OA) and a Kellgren/Lawrence (K/L) (18) score of at least 4 points in the Authors' institution between 2008 and 2010. The study was approved by the Institutional Review Board and all patients provided their informed consent before surgery.

Demographics and pre-operative radiographic evaluation of limb alignment are resumed on Table I.

Preoperatively, all patients had weight-bearing antero-posterior (AP) and latero-lateral (LL) long film radiographs (19). We assumed a hip-knee-ankle angle (HKA) greater than 180° to be a valgus knee and a HKA less than 180° a varus knee (20). Twelve patients with valgus alignment were excluded from the study to avoid data dispersion due to the non-normal distribution of limb-alignment in patients with OA and because of kinematics abnormalities in patients with valgus knees (21-25).

Acquisition protocol

A surgical navigation system (BLU-IGS, Orthokey, Lewes, Delaware) (26) and a specific software (KLEE, Orthokey, Lewes, Delaware) (27) were used to acquire anatomical landmarks and passive joint kinematics (28). Anatomical and kinematic data were collected by the two Senior Authors after medial parapatellar arthrotomy before anterior cruciate ligament (ACL) and meniscal removal and after cementing final implant. Data were analyzed off-line using the Matlab software (Mathworks, Natick, MA, USA). The joint coordinate reference system (JCS) was defined according to Cole et al. (29) and Grood and Suntay (30). Anatomical landmarks acquired during the procedure are shown on Fig. 1. The functional hip joint centre (HJC) was identified through a pivoting motion, as described by Siston et al. (31).

For the femoral anatomical reference system, the proximal-distal (PD) axis was defined with the femoral mechanical axis (32) i.e., the line connecting HJC and the deepest point in the femoral notch (FN), as defined by Bertin (33). The medial-lateral (ML) axis was defined as the surgical transepicondylar axis (TEA) i.e., the line connecting the medial sulcus of the medial epicondyle (FME) and the lateral epicondyle (FLE), defined as the most lateral prominence of the lateral femoral condyle (6-7). The cross product between the PD- and ML-axis was defined as the anterior-posterior (AP) axis (Fig. 1).

For the tibial anatomical reference system, the PD axis was defined as the tibial mechanical axis i.e., the

Table I. *Demographics and pre-operative alignment.*

VARIABLE	VALUE
Sex (M/F)	42/66
Age* (years)	71 ± 4 (62-84)
Limb (right/left)	48/60
BMI* (kg/m2)	29 ± 3 (26-37)
HKA-pre*	174.7° ± 3.4° (168.1°-178.2°)

M**: male; **F**: female; **BMI**: body mass Index; **HKA-pre**: preoperative hip-knee-ankle angle; **HKA-post**: post-operative hip-knee-ankle angle; **:values are expressed as mean±SD with range in brackets.*

line connecting the tibial spine point (TS) and the point equidistant between the medial (MM) and the lateral (LM) malleoli. The AP-axis was defined as the projection of tibial tuberosity point (TT) to the PD-axis, and the ML-axis, as the cross product between PD-axis and ML-axis (28) (Fig. 1).

FFA acquisition

The knee was cycled through three complete passive range of motions (PROM), from full extension to 120° of flexion and back to full extension, before and after implant positioning.

For each patient, the pre-operative FFA acquisition was performed after medial parapatellar arthrotomy, with intact menisci and ACL, using a temporary suture repair to reduce the patella in its anatomical position. In all acquisitions, the movement was performed while maintaining the femur elevated with one hand and holding the foot in neutral position with the other hand, without superimposing any additional load (26, 28).

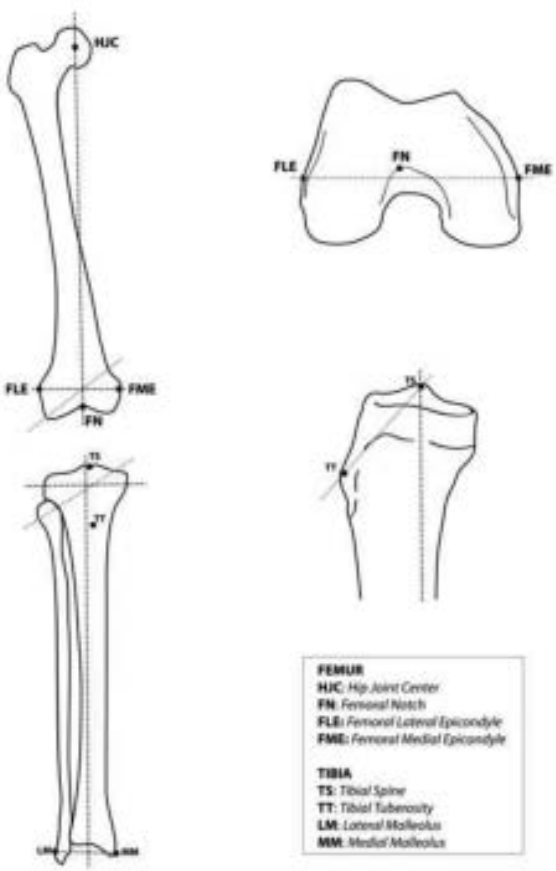


Fig. 1. *Anatomical landmarks acquired and joint coordinate reference system with femoral and tibial PD, ML and AP axis.*

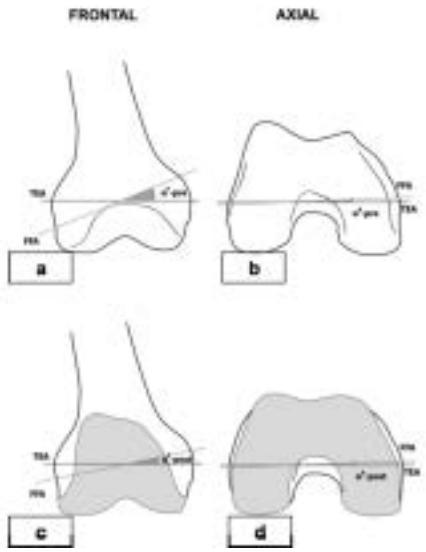


FIG. 2. ***a, b****): The angle between FFA and surgical TEA on frontal and axial plane before TKR implantation;* ***c, d****): illustrates the angle between FFA and surgical TEA after final implant positioning; The drawing illustrates a significant modification of FFA position with respect to TEA only on frontal plane and underlines that pre-operative FFA is in a more varus position respect to TEA.*

The FFA was computed using the mean helical axis algorithm (34). The angle between FFA and surgical TEA was determined on frontal (α^F-pre) and axial (α^A-pre) plane (Fig. 2).

Navigation was used to determine implant positioning. Femoral component rotational positioning was determined averaging Whiteside line and surgical TEA (4, 5) without considering PCA. After final implant positioning and capsular closure, post-operative FFA acquisition was performed, repeating the same movement and FFA was again computed. The angle between FFA and surgical TEA was again determined on frontal (α^F-post) and axial (α^A-post) plane (Fig. 2).

A varus position of FFA, with respect to surgical TEA in the frontal plane, was assigned a negative value, while a valgus alignment of FFA to TEA was assigned a positive valgus. For axial plane, we assumed negative values to describe a more internally rotated position of FFA with respect to surgical TEA, while more externally rotated position of FFA with respect to surgical TEA was assigned positive values (Fig. 2).

Data Analysis

The Grood and Suntay algorithm (30) was used to decompose instantaneous rotations and displacements, in order to describe the relative motion of the tibial

Table II. *Pre-operative and post-operative angles between FFA and TEA on frontal and axial plane and limb alignment.*

VARIABLE	PRE-OP	POST-OP	Δ	p	COMMENT
αF#	-3.9° ± 5.9°	-0.7° ± 5.8°	3.2° ± 11.7°	**<0.0001**	**REDUCTION OF VARUS POSITION RESPECT TO TEA ON FRONTAL PLANE**
αA#	0.8° ± 5.6°	0.0° ± 4.6°	0.8° ± 10.2°	0.0539	NOT SIGNIFICANT INCREASED INTERNAL ROTATION RESPECT TO TEA ON AXIAL PLANE
HKA*	174.7° ± 3.4° (168.1°-178.2°)	179.3° ± 2.4° (178.9°-184.5°)	4.6° ± 5.8°	**<0.0001**	**REDUCTION OF VARUS ALIGNMENT TO HAVE A NEUTRAL MECHANICAL ALIGNMENT OF TKR**

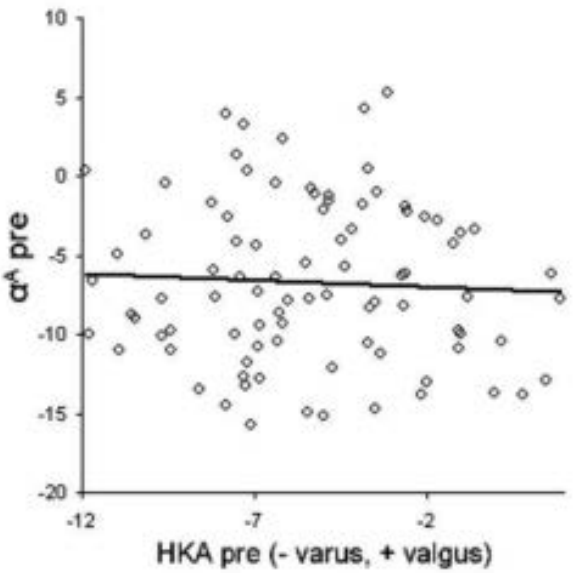
Pre-op**: pre-operative; **Post-op**: post-operative; **Δ**: variation; **p**: significancy; **αF**: angle between functional flexion axis and surgical transepicondylar line on frontal plane; **αA** (TEA: trans-epicondylar axis): angle between functional flexion axis and surgical transepicondylar line on axial plane; **HKA**: hip-knee-ankle angle; **#**: values are expressed as mean±SD; **: values are expressed as mean±SD with range in parentheses.*

frame with respect to the femoral one. Knee kinematics during PROM were described by computing the means of instantaneous flexion–extension (FE), internal-external (IE) and varus–valgus (VV) rotations as the cardian decomposition in the JCS.

Starting from the instantaneous helical axes (IHA), elaborated for each PROM using the least square approach (35), the mean helical axis (MHA) was computed and defined as the functional flexion axis (FFA) (34).

Statistical analysis

Descriptive statistical analysis was performed to evaluate data distribution both on pre- and post-implant values. Angles were described by means and standard deviations. The paired Student's *t*-test was performed to investigate whether a correlation exists between α^F-pre and α^F-post and between α^A-pre and α^A-post. The Pearson correlation was used to test if a relationship exists between α^F and α^A, before and after implant positioning, and pre-operative limb alignment determined with the HKA angle. Statistical significance was set to 95% (P=0.05) for all the tests.



Fg. 3. *Chart shows no correlation between pre-operative limb alignment and preoperative FFA on axial plane.*

RESULTS

The post-operative FFA after TKR was different from the pre-operative FFA only on frontal plane. No significant difference was found between pre-operative FFA and post-operative FFA on axial plane in a 0° to 120° PROM (Tab. II). The angle between pre-operative FFA and surgical TEA was -3.9°±5.9° on frontal plane (α^F-pre) and 0.8°±5.6° on axial plane (α^A-pre) (Fig. 2, Table II).

No correlation was found between HKA-pre and α^A-pre (Fig. 3; Tab. III), while a significant correlation was found between HKA-pre and α^F–pre

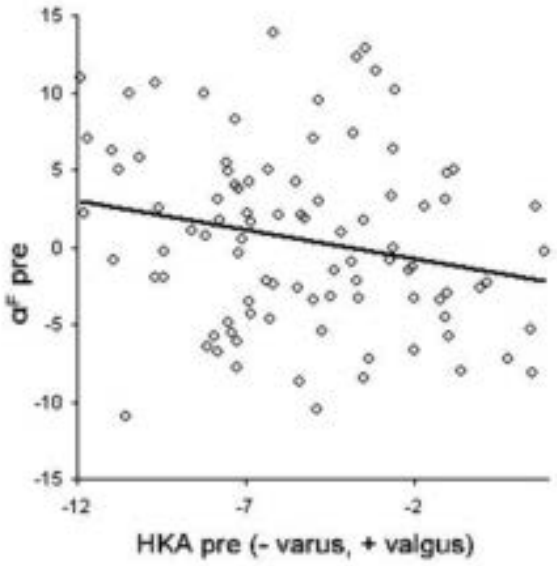


Fig. 4. *Chart showing a linear correlation between limb alignment and pre-operative FFA on frontal plane.*

(Fig. 4; Tab. III).

The value of α^F-post was -0.7°±5.8° and the value of α^A-post was 0°±4.6° (Table II, III). No correlation was found between HKA-pre and α^F-post or α^A-post (Tab. III). Pre- and post-operative values of limb alignment are reported on Table II.

DISCUSSION

The optimal surgical reference to optimize rotational positioning of the femoral component during a TKR is still debated. Navigation has been suggested to improve accuracy of implant positioning and to reduce the number of outliers in frontal and axial alignment (9-12).

Nevertheless, previous studies (17, 36) have demonstrated poor reproducibility of navigation when the TEA is used as a reference to set femoral component rotation. Conversely, other reports (13, 27) have demonstrated that TEA remains the gold standard among anatomical landmarks.

Recently, kinematically derived knee flexion axis has been suggested as a more reproducible and reliable reference to optimize femoral component positioning in TKR (17, 37-39) over conventional anatomical references. To the best of our knowledge, no study has demonstrated a correlation between kinematically derived FFA and conventional surgical TEA reference in navigated TKR. Therefore, the purpose of the present study was to investigate whether pre-operative FFA of the knee in patients with end-stage OA and varus alignment changes after TKR. The secondary purpose was to determine whether a correlation exists between pre- and post-operative FFA and the amount of pre-operative varus deformity.

Our study has several limitations. 1): valgus

Table III. *Correlation of αF-pre, αF-post, αA-pre and α-A-post with pre-operative alignment.*

VARIABLE	VALUE	HKA PRE-OP*	p	COMMENT
αF-pre#	-3.9° ±5.9°	174.7° ± 3.4° (168.1°-178.2°)	**0.0445**	**PRE-OPERATIVE FFA POSITION ON FRONTAL PLANE IS MORE VARUS RESPECT TO TEA IN PATIENTS WITH HIGHER VARUS ALIGNMENT**
αA-pre#	0.8° ±5.6°		0.9825	PRE-OPERATIVE FFA POSITION ON AXIAL PLANE NOTINFLUENCED BY AMOUNT OF VARUS DEFORMITY
αF-post#	-0.7° ±5.8		0.9698	POST-OPERATIVE FFA POSITION ON FRONTAL AND AXIAL PLANE NOT INFLUENCED BY AMOUNT OF VARUS DEFORMITY
α-A-post#	0° ± 4.6°		0.9472	

HKA PRE-OP*: pre-operative hip-knee-ankle angle (TEA: trans-epicondylar axis);* ***p****: variation;* ***αF-pre****: preoperative angle between functional flexion axis and surgical transepicondylar line on frontal plane;* ***αA-pre****: pre-operative angle between functional flexion axis and surgical transepicondylar line on axial plane;* ***αF-post****: post-operative angle between functional flexion axis and surgical transepicondylar line on frontal plane;* ***αA-post****: post-operative angle between functional flexion axis and surgical transepicondylar line on axial plane; #: values are expressed as mean ± SD; *: values are expressed as mean ± SD with range in parentheses.*

patients were not included. As suggested by Akagi et al. (40), in valgus knees a hypoplasia of the lateral condyle exists and modifies the femoral rotation throughout the ROM. Further investigation is going on to have a sufficient sample size of patients with primary OA and pre-operative valgus deformity; 2): FFA position was referred to the position of the surgical TEA on both frontal and axial plane. No CT scan was used to confirm surgical TEA position with respect to anatomical and ct-TEA. The comparison between surgical, anatomical and CT-TEA was not a purpose of the present study. Nevertheless, surgical TEA is widely accepted as gold standard for intra-operative rotational positioning of the femoral component with a non minimally-invasive approach, with good direct visualization of the epicondyles (7, 25, 41-45); 3): no comparison was performed with other anatomical landmarks used for rotational positioning of the femoral component during TKR i.e., the posterior condylar axis (PCA), the Whiteside's Line (5) and the anterior trochlear line (4, 46). Further investigation is needed to compare FFA with different rotational landmarks for femoral component positioning; 4): no comparison between different prosthetic designs was performed. Using a posterior stabilized (PS) design or a fixed bearing implant could have lead to different results; 5): pre-operative FFA acquisition was performed after medial parapatellar capsulotomy. Oussedik et al. (39) and Doro et al. (17) have already demonstrated that FFA is not influenced by this surgical exposure. Further investigation is needed to underline a possible contribution of capsule on FFA of the native knee; 6): no comparative study with an age matched control group with normal knees was performed. This is an intrinsic limitation of using a navigation system requiring rigid pins fixed to the bone. Colle et al. (37) have previously demonstrated that OA affects knee kinematics only on frontal plane comparing OA patients with an ACLR control group, that can be assumed to have a kinematic similar to a normal knee (47-49).

The present study supports these findings, since a difference between pre- and post-operative FFA was demonstrated only on frontal plane.

Considering three dimensional lower limb alignment also on sagittal and axial plane could have lead to different results. As already underlined by Oussedik et al (39), kinematically determined FFA could be influenced by tourniquet use and absence of active muscle contraction in the patient under anaesthesia. Further investigation is needed to evaluate the contribution of active muscle contraction to knee kinematics.

We did not analyse different flexion ranges separately, where the knee joint could present different motion patterns, i.e. the "screw-home" mechanism (50-52). Siston et al. (53) previously found a reduced screw-home motion in TKA patients respect to the pre-operative OA knees and an abnormal varus-valgus rotation in early flexion using a PS design. Further data analysis could reveal if different flexion ranges require different finite helical axis to describe the relative motion of the femur and the tibial.

Ligament release was not quantified and final FFA acquisition was performed after that ligament balancing was considered optimal by the operating surgeon.

Despite these limitations, to the best of our knowledge, the present study is the first report to demonstrate *in vivo* that TKR modifies FFA in patients with end stage OA only on frontal plane, while no difference was found between pre- and post-operative FFA on axial plane.

The angle between pre-operative FFA and TEA was -3.9°±5.9°, underlining that pre-operative FFA is in a more varus position respect to TEA. Moreover, no correlation was found between HKA-pre and α^A-pre while a correlation was found between HKA-pre and α^F-pre. The present study does not confirm the findings of Asano et al. (6) who determined a fixed functional axis corresponding to the surgical TEA during a 0° to 90° flexion in 9 healthy knees using a biplanar image-matching technique. Knee OA has been defined an "extension-gap disease" by Repicci et al. (54, 55) to underline that cartilage and subchondral bone erosion are mainly present on the weight-bearing surface of the medial femoral condyle.

Post-operative FFA position on frontal plane may be influenced by this wear pattern and by neutral mechanical alignment of the implant. On axial plane, the absence of wear on the posterior aspect of the medial femoral condyle and its circular shape are not altered by femoral component implantation. Whether a complete restoration of a neutral mechanical alignment is the optimal solution is a matter of debate (56-61). Further investigation is needed to determine whether a kinematic alignment of current prosthetic designs (58) or different implants with asymmetrical surfaces for the medial and lateral condyles are viable options to improve TKR kinematics.

In conclusion, the present study has demonstrated that the position of FFA on frontal plane is dependent on limb alignment and that TKR modifies the

position of FFA only on frontal plane. The position of FFA on axial plane is not dependent on the amount of varus deformity and is not influenced by TKR.

REFERENCES

1. Romero J, Stähelin T, Binkert C, Pfirrmann C, Hodler J, Kessler O. The clinical consequences of flexion gap asymmetry in total knee arthroplasty. J Arthroplasty 2007; 22(2):235-40.
2. Verlinden C, Uvin P, Labey L, Luyckx JP, Bellemans J, Vandenneucker H. The influence of malrotation of the femoral component in total knee replacement on the mechanics of patellofemoral contact during gait: an in vitro biomechanical study. J Bone Joint Surg Br 2010; 92(5):737-42.
3. Laskin RS. Flexion space configuration in total knee arthroplasty. J Arthroplasty 1995;10(5):657-660.
4. Victor J. Rotational alignment of the distal femur: a literature review. Orthop Traumatol Surg Res 2009; 95(5):365-72.
5. Arima J, Whiteside LA, McCarthy DS, White SE. Femoral rotational alignment, based on the anteroposterior axis, in total knee arthroplasty in a valgus knee. A technical note. J Bone Joint Surg Am 1995; 77(9):1331-4.
6. Asano T, Akagi M, Nakamura T. The functional flexion-extension axis of the knee corresponds to the surgical epicondylar axis: *in vivo* analysis using a biplanar image- matching technique. J Arthroplasty 2005; 20(8):1060-7.
7. Berger RA, Rubash HE, Seel MJ, Thompson WH, Crossett LS. Determining the rotational alignment of the femoral component in total knee arthroplasty using the epicondylar axis. Clin Orthop Relat Res 1993; 286:40-7.
8. Poilvache PL, Insall JN, Scuderi GR, Font-Rodriguez DE. Rotational landmarks and sizing of the distal femur in total knee arthroplasty. Clin Orthop Relat Res. 1996; 331:35-46.
9. Bäthis H, Perlick L, Tingart M, Lüring C, Zurakowski D, Grifka J. Alignment in total knee arthroplasty. A comparison of computer-assisted surgery with the conventional technique. J Bone Joint Surg Br 2004; 86(5):682-7.
10. Decking R, Markmann Y, Fuchs J, Puhl W, Scharf HP. Leg axis after computer- navigated total knee arthroplasty: a prospective randomized trial comparing computer-navigated and manual implantation. J Arthroplasty 2005; 20(3):282-8.
11. Sparmann M, Wolke B, Czupalla H, Banzer D, Zink A. Positioning of total knee arthroplasty with and without navigation support. A prospective, randomised study. J Bone Joint Surg Br 2003; 85(6):830-5.
12. Stulberg SD, Loan P, Sarin V. Computer-assisted navigation in total knee replacement: results of an initial experience in thirty-five patients. J Bone Joint Surg Am 2002; 84(2):90-8.
13. Siston RA, Patel JJ, Goodman SB, Delp SL, Giori NJ. The variability of femoral rotational alignment in total knee arthroplasty. J Bone Joint Surg Am 2005; 87(10):2276-80.
14. Siston RA, Giori NJ, Goodman SB, Delp SL. Intraoperative passive kinematics of osteoarthritic knees before and after total knee arthroplasty. J Orthop Res 2006; 24(8):1607-14.
15. Hoppe S, Mainzer JD, Frauchiger L, Ballmer PM, Hess R, Zumstein MA. More accurate component alignment in navigated total knee arthroplasty has no clinical benefit at 5-year follow-up. Acta Orthop 2012; 83(6):629-33.
16. Burnett RS, Barrack RL. Computer-assisted total knee arthroplasty is currently of no proven clinical benefit: a systematic review. Clin Orthop Relat Res 2013; 471(1):264-76.
17. Doro LC, Hughes RE, Miller JD, Schultz KF, Hallstrom B, Urquhart AG. The reproducibility of a kinematically-derived axis of the knee versus digitized anatomical landmarks using a knee navigation system. Open Biomed Eng J 2008; 10(2):52-6.
18. Kellgren JH, Lawrence JS. Radiological assessment of osteo-arthrosis. Ann Rheum Dis 1957; 16(4):494-502.
19. LaValley MP, McLaughlin S, Goggins J, Gale D, Nevitt MC, Felson DT. The lateral view radiograph for assessment of the tibiofemoral joint space in knee osteoarthritis: its reliability, sensitivity to change, and longitudinal validity. Arthritis Rheum 2005; 52(11):3542-7.
20. Cooke TD, Li J, Scudamore RA. Radiographic assessment of bony contributions to knee deformity. Orthop Clin North Am 1994; 25(3):387-93.
21. Deakin AH, Basanagoudar PL, Nunag P, Johnston AT, Sarungi M. Natural distribution of the femoral mechanical-anatomical angle in an osteoarthritic population and its relevance to total knee arthroplasty. Knee 2012; 19(2):120-3.
22. Eckhoff DG. Effect of limb malrotation on

malalignment and osteoarthritis. Orthop Clin North Am 1994; 25(3):405-14.
23. Weidow J, Kärrholm J, Saari T, McPherson A. Abnormal motion of the medial femoral condyle in lateral knee osteoarthritis. Clin Orthop Relat Res 2007; 454:27-34.
24. Whalen J. The anatomy and functional axes of the femur. J Bone Joint Surg Am 988; 70(1):151-2.
25. Yoshioka Y, Siu D, Cooke TD. The anatomy and functional axes of the femur. J Bone Joint Surg Am 1987; 69(6):873-80.
26. Martelli S, Zaffagnini S, Bignozzi S, Lopomo NF, Iacono F, Marcacci M. KIN-Nav navigation system for kinematic assessment in anterior cruciate ligament reconstruction: features, use, and perspectives. Proc Inst Mech Eng H 2007; 221(7):725-37.
27. Martelli S, Lopomo N, Greggio S, Ferretti E, Visani A. Development and applications of a software tool for diarthrodial joint analysis. Comput Methods Programs Biomed 2006; 83(1):50-6.
28. Martelli S, Zaffagnini S, Bignozzi S, Bontempi M, Marcacci M. Validation of a new protocol for computer-assisted evaluation of kinematics of double-bundle ACL reconstruction. Clin Biomech 2006; 21(3):279-87.
29. Cole GK, Nigg BM, Ronsky JL, Yeadon MR. Application of the joint coordinate system to three-dimensional joint attitude and movement representation: a standardization proposal. J Biomech Eng 1993; 115:344-9.
30. Grood ES, Suntay WJ. A joint coordinate system for the clinical description of three- dimensional motions: application to the knee. J Biomech Eng 1983; 105:136-144.
31. Siston RA, Delp SL. Evaluation of a new algorithm to determine the hip joint center. J Biomech 2006; 39(1):125-30.
32. Gamage SS, Lasenby J. New least squares solutions for estimating the average centre of rotation and the axis of rotation. J Biomech 2002; 35(1):87-93.
33. Bertin KC. Intramedullary instrumentation for total knee arthroplasty. P175 in Goldberg V (editor); Controversies of total knee arthroplasty. Raven Press New York 1991; Presented at the Zimmer Orthopaedic Symposium, Scottsdale, AZ, 1989.
34. Sheehan FT. The finite helical axis of the knee joint (a non-invasive *in vivo* study using fast-PC MRI). J Biomech 2007; 40(5):1038-47.
35. Woltring HJ, Huiskes R, de Lange A, Veldpaus FE. Finite centroid and helical axis estimation from noisy landmark measurements in the study of human joint kinematics. J Biomech 1985; 18(5):379-89.
36. Jenny JY, Boeri C. Low reproducibility of the intra-operative measurement of the transepicondylar axis during total knee replacement. Acta Orthop Scand 2004; 75(1):74-7.
37. Colle F, Bignozzi S, Lopomo N, Zaffagnini S, Sun L, Marcacci M. Knee functional flexion axis in osteoarthritic patients: comparison *in vivo* with transepicondylar axis using a navigation system. Knee Surg Sports Traumatol Arthrosc 2012; 20(3):552-8.
38. Matziolis G, Pfiel S, Wassilew G, Boenicke H, Perka C. Kinematic analysis of the flexion axis for correct femoral component placement. Knee Surg Sports Traumatol Arthrosc 2011; 19(9):1504-9.
39. Oussedik S, Scholes C, Ferguson D, Roe J, Parker D. Is femoral component rotation in a TKA reliably guided by the functional flexion axis? Clin Orthop Relat Res 2012; 470(11):3227-32.
40. Akagi M, Yamashita E, Nakagawa T, Asano T, Nakamura T. Relationship between frontal knee alignment and reference axes in the distal femur. Clin Orthop Relat Res 2001; 388:147-56.
41. Mantas JP, Bloebaum RD, Skedros JG, Hofmann AA. Implications of reference axes used for rotational alignment of the femoral component in primary and revision knee arthroplasty. J Arthroplasty 1992; 7(4):531-5.
42. Matsuda S, Matsuda H, Miyagi T, Sasaki K, Iwamoto Y, Miura H. Femoral condyle geometry in the normal and varus knee. Clin Orthop Relat Res 1998; 349:183-8.
43. Matziolis G, Krocker D, Weiss U, Tohtz S, Perka C. A prospective, randomized study of computer-assisted and conventional total knee arthroplasty. Three-dimensional evaluation of implant alignment and rotation. J Bone Joint Surg Am 2007; 89(2):236-43.
44. Olcott CW, Scott RD. The Ranawat Award. Femoral component rotation during total knee arthroplasty. Clin Orthop Relat Res 1999; 367:39-42.
45. Stiehl JB, Abbott BD. Morphology of the transepicondylar axis and its application in primary and revision total knee arthroplasty. J Arthroplasty 1995; 10(6):785-9.
46. Morizane K, Takahashi T, Konishi F, Yamamoto H. The anterior trochlear line as a reference for femoral component positioning in total knee arthroplasty.

Knee Surg Sports Traumatol Arthrosc 2011; 19(12):2009-15.
47. Georgoulis AD, Papadonikolakis A, Papageorgiou CD, Mitsou A, Stergiou N. Three-dimensional tibiofemoral kinematics of the anterior cruciate ligament-deficient and reconstructed knee during walking. Am J Sports Med 2003; 31(1):75-9.
48. Kanisawa I, Banks AZ, Banks SA, Moriya H, Tsuchiya A. Weight-bearing knee kinematics in subjects with two types of anterior cruciate ligament reconstructions. Knee Surg Sports Traumatol Arthrosc 2003; 11(1):16-22.
49. Papannagari R, Gill TJ, Defrate LE, Moses JM, Petruska AJ, Li G. *In vivo* kinematics of the knee after anterior cruciate ligament reconstruction: a clinical and functional evaluation. Am J Sports Med 2006; 34(12):2006-12.
50. Freeman MA, Pinskerova V. The movement of the normal tibio-femoral joint. J Biomech 2005; 38(2):197-208.
51. Moglo KE, Shirazi-Adl A. Cruciate coupling and screw-home mechanism in passive knee joint during extension-flexion. J Biomech 2005; 38(5):1075-83.
52. Piazza SJ, Cavanagh PR. Measurement of the screw-home motion of the knee is sensitive to errors in axis alignment. J Biomech 2000; 33(8):1029-34.
53. Siston RA, Cromie MJ, Gold GE, Goodman SB, Delp SL, Maloney WJ, Giori NJ. Averaging different alignment axes improves femoral rotational alignment in computer-navigated total knee arthroplasty. J Bone Joint Surg Am 2008; 90(10):2098-104.
54. Repicci JA. Mini-invasive knee unicompartmental arthroplasty: bone-sparing technique. Surg Technol Int 2003; 11:282-6.
55. Repicci JA, Hartman JF. Minimally invasive unicondylar knee arthroplasty for the treatment of unicompartmental osteoarthritis: an outpatient arthritic bypass procedure. Orthop Clin North Am 2004; 35(2):201-16.
56. Bellemans J. Neutral mechanical alignment: a requirement for successful TKA: opposes. Orthopedics 2011;34(9):e507-9.
57. Bellemans J, Colyn W, Vandenneucker H, Victor J. The Chitranjan Ranawat award: is neutral mechanical alignment normal for all patients? The concept of constitutional varus. Clin Orthop Relat Res 2012; 470(1):45-53.
58. Howell SM, Howell SJ, Kuznik KT, Cohen J, Hull ML. Does a kinematically aligned total knee arthroplasty restore function without failure regardless of
59. Johnson AJ, Harwin SF, Krakow KA, Mont MA. Alignment in Total Knee Arthroplasty: Where Have We Come From and Where Are We Going? Surg Technol Int. 2011; XXI:183-188.
60. Magnussen RA, Weppe F, Demey G, Servien E, Lustig S. Residual varus alignment does not compromise results of TKAs in patients with preoperative varus. Clin Orthop Relat Res. 2011;469(12):3443-50.
61. Parratte S, Pagnano MW, Trousdale RT, Berry DJ. Effect of postoperative mechanical axis alignment on the fifteen-year survival of modern, cemented total knee replacements. J Bone Joint Surg Am. 2010;92(12):2143-9.

PAIN MANAGEMENT AFTER TOTAL KNEE ARTHROPLASTY: THE GOOD, THE BAD AND THE UGLY

A. SPINARELLI, L. MORETTI, G. MARELLA, G. SOLARINO, G. MACCAGNANO and B. MORETTI

Department of Neuroscience and Organs of Sense, Orthopaedic and Traumatology Unit, University of Bari, General Hospital, Bari, Italy

Improvement in pain management after knee replacement surgery has made progress in the last years, improving the results of this type of operation. Among these techniques, multimodal have shown the best results. In this study we try to compare the results of a combination of intravenous analgesia (IA), oral controlled analgesia (OCA) and periarticular injection (PAI) with our traditional protocol consisting in intravenous analgesia and femoral nerve block (IA/FNB). One-hundred patients, undergoing primary unilateral total knee arthroplasty between June 2014 and June 2015 were randomized into 2 groups. Mean patient age was 69.4. The first group received the intravenous analgesia combined with continuous femoral nerve block, while the second group received the new combined protocol. We used the same technique with standard medial parapatellar approach for all patients and they all received pre-emptive analgesia and postoperative pain protocols. All patients were interviewed daily postoperatively at 3 days, at discharge and at 3 months. The 2 groups had a similar discharge period (traditional group 7.3 days, combined group 6.9 days). In both groups, the results indicated no statistical difference in regards to rest and continuous passive movement. Pain on ambulation was the only category that was statistically lower in the PAI/IA/OCA group compared to traditional group.

Routine knee replacement surgery is burdened with important postoperative pain. This pain affects the patient emotionally and affects functional recovery (1). Good pain control increases patient satisfaction in the post operative period, helps functional recovery and decreases hospitalization (2, 3, 4).

Early rehabilitation with little pain decreases the risk of complications such as DVT, PE, urinary retention and respiratory complications. The post-operative analgesia in this type of surgery is usually multimodal and includes various methods: epidural analgesia, opioids, peripheral nerve blocks, intra-articular and intrasynovial opioids, oral analgesics and local anaesthetics (5, 6, 7).

Multimodal pain management is the use of multiple agents that act at different sites on the pain pathway (8). Over the last year, our multimodal pain management protocol after TKA has undergone several changes using various agents to reduce postoperative pain, including the adoption of pre-emptive analgesia, intravenous analgesia, femoral nerve block, the use of local periarticular injections and the introduction of a comprehensive postoperative pain protocol (9, 10).

The purpose of this study was to compare the results of an oral combined analgesia (OCA), intravenous analgesia (IA) and periarticular injection (PAI) to a combination of IA and femoral nerve block (FNB) in a prospective, randomized control trial.

Key words: TKA, pain management, multimodal approach

Mailing address: Dr Antonio Spinarelli,
Department of Neuroscience and Organs of Sense,
Orthopaedic and Traumatology Unit,
University of Bari, 11 Piazza Giulio Cesare,
Bari 70124, Italy
Tel.: +39 080 5592719 Fax: +39 080 5592713
e-mail: antoniospinarelli@gmail.com

0393-974X (2015)

MATERIALS AND METHODS

The local Ethics Committee approved the trial and all patients gave written informed consent. Prior to surgery, patients were informed about the surgical anaesthetic method they would receive and about the study and post-operative processes and written consent was obtained.

Patients were included if they had to be operated on for unilateral knee replacement with a diagnosis of primary knee OA and agreed to participate in the study and respecting the procedures. The exclusion criteria was: American Society of Anaesthesiologists (ASA) grades IV/V, liver dysfunction, liver disease, kidney disease, severe heart failure, obesity (body mass index>40, neuropathic pain, allergy to local anaesthetics.

Patients with deformities >15° of varus, valgus or flexion contracture were excluded. All operations were performed under pneumatic tourniquet by the same surgical team, using the standard anterior median longitudinal skin incision and medial parapatellar approach. The same prosthesis (Nexgen® Knee System-Posterior Stabilized; Zimmer®Warsav, Indiana, USA) was used in all patients.

All patients received similar pre-emptive analgesia and spinal anaesthesia with 25G needle and 10-12 mg levobupivacaine and postoperative pain protocols.

100 patients (100 knees) undergoing primary unilateral TKA between June 2014 and June 2015 were included in the study. The 2 groups were demographically similar for sex, age, body mass index and race.

The study group included 42 men and 58 women with a mean age of 69.4 years (range, 58-85 years). Patients were randomized into 2 groups: the first group (A) received the IA/FNB protocol, and the second group (B) received the PAI/IA/OCA. All patients were interviewed daily postoperatively for the first 3 days, at discharge and at 3 months. Time for discharge criteria was recorded, including adequate pain control without significant side effects, stable vital signs and achievement of physical therapy criteria. A validated postoperative numeric rating pain scale (VAS) was used to determine the level of pain.

Data were statistically analysed using SPSS software (v. 10.0; SPSS Inc. Chicago, IL, USA). Student's *t*-test, Mann-Whitney U-test, and $\chi 2$ test were used for comparisons. Significance was set at $p<0.05$.

A pre-emptive analgesic regimen consisting of 1 gr acetaminophen, 50 mg tramadol, 8mg Dexamethasone and 1gr tranexamic acid (for bleeding) was administered to each patient within 1 hour pre-operatively.

In post-op for A, the protocol for FNB included a bolus of 25 cc levobupivacaine. The IA consisted of an elastomeric pump with 40 mg morphine, 120 mg ketorolac and 20 mg metoclopramid for 48 hours. From the third day to discharge pain was treated on demand with acetaminophen 1 gr.

The second group received intraoperative injections (PAI) administered prior to cementation. The cocktail included a combination of 150 mg levobupivacaine and 2g tranexamic acid (for bleeding). This was injected in posteromedial structures, lateral structure patellar

Table I. *Post-operative pain and rest, continue passive motion and ambulation (VAS).*

	Group A	Group B	p
Pre-op	8.16±0.73	8.43±0.32	0.26
Rest			
I	3.52±1.41	2.21±1.26	0.111
II	2.12±0.73	0.96±0.92	0.293
III	1.79±0.59	0.44±0.87	0.94
CPM			
I	5.63±1.27	5.21±1.32	0.19
II	5.21±1.07	4.74±1.19	0.112
III	3.57±0.97	3.60±1.12	0.58
Ambulation			
I	5.10±1.50	4.70±1.89	**000**
II	4.87±1.26	3.87±1.81	**000**
III	4.20±1.19	2.91±1.03	**000**
3 Months	1.12±0.57	1.09±0.63	0.40

and quadriceps tendon, the periarticular synovium, the posterior capsule, anteromedial capsule, periosteum, iliotibial band and subcutaneous plane. It was not injected in the posterolateral corner to prevent injury of the peroneal nerve.

Next step consisted of the same IA like group A. After 48 hours and until 7 days post-op, OCA consisted of 10 mg oxycodone controlled release twice daily, 1 gr acetaminophen twice daily and ketorolac intravenously (only for breakthrough pain).

All patients received 4000 IU of low-molecular-weight heparin for deep vein thrombosis prophylaxis from 12 hours post-surgery for a total of 35 days.

RESULTS

At 3-months follow-up no infections or complications occurred and no reoperations were necessary. No statistical difference ($P<05$) existed for time of discharge between the 2 groups (IA/FNB group 7.3 days, IA/OCA/PAI group 6.9 days). Numeric pain rating scales were statistically lower in the IA/OCA/PAI group on postoperative day 1-2-3 and at discharge for pain on ambulation. However, for every other category (rest, continuous passive motion) on postoperative day 1 and on postoperative day 2, 3 and discharge, both groups were statistically similar ($P<05$).

At 3-month follow-up, no statistical differences existed in regards to pain on ambulation and rest (Table I).

DISCUSSION

Post-operative pain control is an important aspect in the field of orthopedic surgery, especially in total knee replacement, especially in relation to the rehabilitation phase. An acute postoperative pain not adequately treated leads to homeostatic alterations and cardiovascular, kidney and respiratory complications, as well as anxiety, changes in sleep patterns and mental disorders (11, 12). Adequate treatment of postoperative pain contributes significantly to the reduction of perioperative morbidity, measured as incidence of postoperative complications and hospital days and costs, especially in patients at high risk (ASA III-V), that undergo major surgery (13, 14).

The transmission of pain impulses takes place through various chemical mediators and neural structures involved in pain perception, for this reason the pain control can be applied in a satisfactory manner with the use of pharmacological substances with different mechanisms of action (15, 16). Multimodal therapy is therefore based on the synergy of different drugs and different methods of administration, combining opioid analgesics with non-opioid analgesics (NSAIDs, acetaminophen) and other classes of drugs, steroids and local anaesthetics administered neuraxial or perineural and pre-emptive analgesia (17). Pharmacologically we speak of enhancement synergy, where two drugs act on different receptors in which the effect obtained is greater than the sum of the effects of the two drugs individually (18). This phenomenon can result in a reduction of the dosage of the two drugs and also a significant reduction in side effects. We know that the simultaneous use of nonsteroidal anti-inflammatory, COX2, acetaminophen and corticosteroids reduce the opioid dose needed to achieve effective control of postoperative pain.

A common option for perioperative pain control is intravenous analgesia (intravenous pump) (20). Several patients who experienced rebound pain after pump removal negatively influenced our initial experience with a 24 h intravenous pump infusion. As a result, we extended the duration of the intravenous pump infusion to 48 h. A combination of intravenous pump infusion and FNB with or without oral controlled analgesia was later used. However, these techniques led to several side effects including nausea, vomiting, ileus, urinary retention, pruritus, hypotension, bradycardia, cognitive changes and quadriceps weakness (21). As a result, a perioperative multimodal approach was designed to optimize narcotic usage.

In a randomized clinical trial, Busch, et al. demonstrated that a periarticular intraoperative injection containing ropivacaine, ketorolac, epimorphine and epinephrine decreased visual analogue scores for pain and increased scores for patient satisfaction after TKA (22, 23). Furthermore, their technique reduced the use of PCEA postoperatively. Milani, et al. suggest that periarticular anesthetic protocol with a ropivacaine 1% 20mL additive is not superior to oral and intravenous analgesia in patients undergoing TKA

in post-operative pain control, operated limb edema reduction and post-operative mobility improvement (6). Kutzner, et al. recommended perioperative treatment with an intra-articular catheter system that is an easy technique and ensures a markedly faster ambulation following TKA, compared to the treatment with continuous FNB (24).

Our practice has evolved using a multimodal protocol that uses pre-emptive analgesia, local periarticular injections, and a postoperative pain protocol to minimize the dramatic effects of pain. In addition the use of IA/OCA/PAI avoids numerous complications associated with FNB and minimize the risk of postoperative falls that is very high in patients who had FNB. The risk of peripheral neuropaty or toxicity after FNB has been clearly described. (25, 26).

CONCLUSION

The results of this study show that a proper multimodal pain management with intravenous elastomeric pumps, oral analgesics and periarticular injection according to precise patterns could play an important role in the immediate postoperative period after TKA improving patient satisfaction. However the results after three months are similar but patients who received multimodal treatment IA/OCA/PAI have a memory of the surgery more pleasant than the group treated with IA/FNB.

REFERENCES

1. Lee K, Goodman SB. Current state and future of joint replacements in the hip and knee. Expert Rev Med Devices 2008; 5(3):383-93.
2. Barrington JW, Halaszynski TM, Sinatra RS. Perioperative pain management in hip and knee replacement surgery. Am J Orthop 2014; 43(4S):S1-S16.
3. Kallio PJ, Nolan J, Olsen AC, Breakwell S, Topp R, Pagel PS. Anesthesia Preoperative Clinic Referral for Elevated Hba1c Reduces Complication Rate in Diabetic Patients Undergoing Total Joint Arthroplasty. Anesthesiology and pain medicine 2015; 5(3):e24376.
4. Voloshin AG, Lyadov KV, Kiryushin DN, Mukutsa IG, Serebryakov AB. [Clinical aspects of the service of acute postoperative pain treatment]. Anesteziol Reanimatol 2015; 60(1):25-9.
5. Toftdahl K, Nikolajsen L, Haraldsted V, Madsen F, Tønnesen EK, Søballe K. Comparison of peri-and intraarticular analgesia with femoral nerve block after total knee arthroplasty: a randomized clinical trial. Acta Orthop 2007; 78(2):172-9.
6. Milani P, Castelli P, Sola M, Invernizzi M, Massazza G, Cisari C. Multimodal analgesia in total knee arthroplasty: a randomized, double-blind, controlled trial on additional efficacy of periarticular anesthesia. J Arthroplasty. 2015; pii:S0883-5403(15)00428-3.
7. Anastase DM, Cionac Florescu S, Munteanu AM, Ursu T, Stoica CI. Analgesic Techniques in Hip and Knee Arthroplasty: From the Daily Practice to Evidence-Based Medicine. Anesthesiol Res Pract 2014; 569319.
8. Nakai T, Tamaki M1, Nakamura T, Nakai T, Onishi A, Hashimoto K. Controlling pain after total knee arthroplasty using a multimodal protocol with local periarticular injections. J Orthop 2013; 10(2):92-4.
9. Liu J, Yuan W, Wang X, Royse CF, Gong M, Zhao Y, Zhang H. Peripheral nerve blocks versus general anesthesia for total knee replacement in elderly patients on the postoperative quality of recovery. Clin Interv Aging 2014; 9:341-50.
10. Wittig-Wells D1, Johnson I, Samms-McPherson J, Thankachan S, Titus B, Jacob A, Higgins M. Does the use of a brief cryotherapy intervention with analgesic administration improve pain management after total knee arthroplasty? Orthop Nurs 2015; 34(3):148-53.
11. Hirakawa Y, Hara M, Fujiwara A, Hanada H, Morioka S. The relationship among psychological factors, neglect-like symptoms and postoperative pain after total knee arthroplasty. Pain Res Manag 2014; 19(5):251-6.
12. Ali A, Sundberg M, Robertsson O, Dahlberg LE, Thorstensson CA, Redlund-Johnell I, Kristiansson I, Lindstrand A. Dissatisfied patients after total knee arthroplasty: A registry study involving 114 patients with 8-13 years of follow-up. Acta Orthop. 2014; 85(3):229-33.
13. Marques EM, Jones HE, Elvers KT, Pyke M, Blom AW, Beswick AD. Local anaesthetic infiltration

for peri-operative pain control in total hip and knee replacement: systematic review and meta-analyses of short-and long-term effectiveness. BMC Musculoskelet Disord. 2014; 15:220.

14. Chincholkar, M. Poor pain control rather than anaesthetic technique influences outcome in total knee arthroplasty. Br J Anaesth 2014; 112(4):764.
15. Benito MJ, Veale DJ, FitzGerald O, van den Berg WB, Bresnihan B. Synovial tissue inflammation in early and late osteoarthritis. Ann Rheum Dis 2005; 64(9):1263-7.
16. Schaible HG. Nociceptive neurons detect cytokines in arthritis. Arthritis Res Ther 2014; 16(5):470.
17. Meftah M, Wong AC, Nawabi DH, Yun RJ, Ranawat AS, Ranawat CS. Pain management after total knee arthroplasty using a multimodal approach. Orthopedics 2012; 35(5):e660-4.
18. Bonnefont J, Daulhac L, Etienne M, et al. Acetaminophen recruits spinal p42/p44 MAPKs and GH/IGF-1 receptors to produce analgesia via the serotonergic system. Mol Pharmacol 2007; 71(2):407-15.
19. Beaupre LA, Johnston DB, Dieleman S, Tsui B. Impact of a preemptive multimodal analgesia plus femoral nerve blockade protocol on rehabilitation, hospital length of stay, and postoperative analgesia after primary total knee arthroplasty: a controlled clinical pilot study. Scientific World Journal 2012; 273821.
20. Ilfeld BM, Mariano ER, Girard PJ, et al. A multicenter, randomized, triple-masked, placebo-controlled trial of the effect of ambulatory continuous femoral nerve blocks on discharge-readiness following total knee arthroplasty in patients on general orthopaedic wards. Pain 2010; 50(3):477-84.
21. Wang, Fen, et al. Ultrasound and nerve stimulator guided continuous femoral nerve block analgesia after total knee arthroplasty: a multicenter randomized controlled study. Revista Brasileira De Anestesiologia 2015; 65(1):14-20.
22. Gill I, Gallagher K, Busch CA. Is peri-articular multimodal drug infiltration in knee arthroplasty safe when used in conjunction with autologous retransfusion drains? Ann R Coll Surg Engl 2010; 92(4):335-7.
23. Elmallah RK, Cherian JJ, Pierce TP, Jauregui JJ, Harwin SF, Mont MA. New and Common Perioperative Pain Management Techniques in Total Knee Arthroplasty. J Knee Surg 2015; (Epub ahead of print).
24. Kutzner KP, Paulini C, Hechtner M, Rehbein P, Pfeil J. Postoperative analgesia after total knee arthroplasty: Continuous intra-articular catheter vs continuous femoral nerve block. Orthopade 2015; 44(7):566-73.
25. Orebaugh SL, Kentor ML, Williams BA. Adverse outcomes associated with nerve stimulator-guided and ultrasound-guided peripheral nerve blocks by supervised trainees: update of a single-site database. Reg Anesth Pain Med 2012; 37(6):577-82.
26. Pennekamp W, Krumova EK, Feigl GP, Frombach E, Nicolas V, Schwarzer A, Maier C. Permanent lesion of the lateral femoral cutaneous nerve after low-volume ethanol 96% application on the lumbar sympathetic chain. Pain Physician 2013; 16(4):391-7.

BIOTECHNOLOGIES AND BIOMATERIALS IN SPINE SURGERY

G. VADALÀ, F. RUSSO, L. AMBROSIO, A. DI MARTINO, R. PAPALIA and V. DENARO

Department of Orthopaedic and Trauma Surgery, University Campus Bio-Medico of Rome, Italy

Over the past few decades, spine disorders have become a major health concern and the number of spinal surgical procedures has been rising significantly. Several biotechnologies and biomaterials are often used in spine surgery to increase the effectiveness of the treatment. In the degenerative spine, when conservative treatment is ineffective the most recommended surgical procedure is decompression followed by spinal fusion. Success rates of spine fusion extensively rely on bone grafts peculiar properties. Autograft has been considered the gold standard to achieve a solid fusion but current research is focused on the development of new biomaterials. Osteoporosis is the main cause of vertebral compression fractures that are significantly associated with pain and disability, especially in the aging population. Vertebral augmentation is a minimally invasive approach in which cement is injected into the vertebral body to stabilize the fracture. New cements are being developed in the clinical scenario with reabsorbable properties and biomechanical features more similar to the native bone. The development of disc regeneration strategies such as nucleus pulposus restoration and annulus fibrosus repair may represent a minimally invasive procedure towards regeneration rather than fusion. Therefore, biomaterials and tissue engineering are fields of growing interest among both surgeons and manufacturing companies, with a major involvement in spine surgery. This review discusses current and novel biotechnologies and biomaterial used in spine surgery employing fusion, augmentation and regeneration.

Over the past few decades, spine disorders have become a major health concern since the number of surgical procedures performed to treat traumatic, degenerative, infective, inflammatory, neoplastic and iatrogenic diseases involving the spine has been raising significantly (1, 2). Thus, several biotechnologies and biomaterials are often used in spine surgery to increase the effectiveness of these surgical procedures.

Generally, in degenerative spine the decision to proceed with surgery is made when conservative treatment is ineffective in relieving back pain or leg pain due to radiculopathy, myelopathy or spinal stenosis. In this regard, the most recommended surgical procedure is decompression followed by spinal fusion. The primary aim of fusion is to obtain a functional and structural unity between affected spinal segments, thence leading to a significant loss of local flexibility. Success rates of spine fusion rely extensively on bone grafts' peculiar properties: to date, autograft has been considered the gold standard to achieve a solid spinal fusion, even though donor site morbidity remains a crucial issue that cannot be disregarded. Current research is focused on the development of new biomaterials that might overcome these disadvantages, while retaining the fundamental features needed to yield spinal arthrodesis.

Key words: spine surgery, fusion, biomaterials, intervertebral disc regeneration, annulus fibrosus repair, vertebral augumentation, stem cells, platelet rich plasma

Mailing address: Gianluca Vadalà, MD, PhD, Department of Orthopedic and Trauma Surgery, Campus Bio-Medico University of Rome, Via Alvaro del Portillo 200, 00128 Rome, Italy
Tel.: +39 06 22 54 19142 Fax: +39 06 22 54 11638

0393-974X (2015)

Osteoporosis is the main cause of vertebral compression fractures that are significantly associated with pain and disability, especially in the aging population. Vertebral augmentation (vertebroplasty or kyphoplasty) is a minimally invasive approach in which cement is injected into the vertebral body to stabilize the fracture. Cement is also utilized to increase the stability of pedicular screws used for spinal instrumentation in the osteoporotic spine. New cements are being developed with reabsorbable properties and biomechanical features more similar to the native bone.

Overall, research in spine surgery is mainly oriented towards the development of disc regeneration strategies such as nucleus pulposus (NP) restoration and annulus fibrosus (AF) repair. Therefore, biomaterials and tissue engineering are of growing interest among both surgeons and manufacturing companies, with a major involvement in spine surgery.

This review will discuss current and novel biotechnologies and biomaterial used in spine surgery employing fusion, augmentation and regeneration.

Spinal Fusion

After spinal decompression is performed, arthrodesis is the fundamental surgical procedure to provide stability of the spine, in furtherance of treatment of diseases affecting primarily facet joints and intervertebral disc (IVD).

Successful outcomes strictly depend upon biocompatible substrates and specifically selected approaches, according to the spinal segment to be fused. Spinal fusion is ultimately accomplished through the implantation of bone grafts into the intervertebral space after discectomy (i.e. interbody fusion), between the transversal processes or beside facet joints (i.e. posterolateral fusion). Fusion sites are then stabilized by specialized instrumentation, in order to achieve a complete and long-lasting arthrodesis.

Spinal fusion healing is a complex process that starts with an inflammatory response triggered by graft incorporation. Fibroblast-like cells then begin to secrete structural proteins and soluble factors that promote angiogenesis and synthesis of a fibrovascular stroma. Growth factors released within the matrix induce chemotaxis and differentiation of mesenchymal stem cells (MSCs) into chondroblasts, which lead to cartilage formation. Vascular invasion marks the transition from the cartilaginous intermediate to new bone tissue through endochondral ossification, driven by osteoblasts. Successively, necrotic tissue and immature bone undergo resorption through the action of osteoclasts, thus forming the new haversian systems of mature cortical bone by a process termed "creeping substitution". Further bone remodeling is characterized by cortical rim thickening, increase in bone marrow activity and formation of secondary spongiosa. The whole process is generally completed in a year (3).

Pseudoarthrosis, which indicates a pathological nonunion of spinal segments causing pain and mechanical instability, is one of the most common complications encountered in spine surgery (4). Pseudoarthrosis rate subsequent to spinal fusion ranges from 5% to 35% and is determined by different variables, such as affected spinal segment (cervical or lumbar), number of involved levels (single or multiple), fusion site (interbody or posterolateral), instrumentation utilized and patient related factors (e.g. age, tobacco use, metabolic disorders etc.) (4).

An ideal bone graft should show three fundamental properties: 1) osteogenesis: ability to stimulate bone formation by osteoblasts activation; 2) osteoinduction: process by which growth factors induce chemotaxis and differentiation of MSCs into osteoblasts; 3) osteoconduction: process by which bone tissue adapts to a surface and grows within its tridimensional structure (5).

Autograft

Autologous bone graft harvested from the iliac crest has historically been considered the gold standard in bone grafting for spinal fusion. Indeed, it yields a remarkable osteo-conductive matrix constituted by type I collagen and hydroxyapatite, which carries osteogenic cells within a growth factors-rich environment.

As an autologous specimen, autograft does not suffer from poor histocompatibility, rejectability or any risk of disease transmission. Moreover, its corticocancellous structure shows suitable biomechanical properties to support physiological loads in spine.

On the other hand, donor site morbidity remains

a major concern being the most likely complication associated with autologous bone grafting. It includes prolonged post-operative pain, hematoma, infection, gait, bruising, ureteral injury, peritoneal perforation, pelvic fracture, vascular and neurological injury, poor cosmesis, increased operative time and blood loss (5).

Overall, a systematic review has shown an average fusion rate of 77% subsequent to autograft implantation (6).

Less commonly, cortical autografts have been derived from the fibula, the clavicle, the manubrium and the ribs, while cancellous substrates can be obtained from Gerdy's tubercle, the distal portion of the radius and the distal portion of the tibia.

Local bone grafting from laminectomy and facetectomy has also been proposed as a valid alternative to avoid donor site complications (5). However, certain studies have reported lower fusion rates with local bone graft compared to autograft due to inadequate cortical/cancellous rate and high content of soft tissues (7).

Allograft

Allogeneic bone graft harvested from cadavers is to date the most common alternative to autograft in spine surgery. However, even if we exclude autografts most notable issues related to donor site morbidity, it suffers from significant disadvantages, such as immunogenicity and risk of disease transmission (especially HBV, HCV and HIV). Donor serologic screening, attentive processing and specialized harvesting techniques are needed to abate these complications, albeit heterogeneity of donor population makes allograft efficacy difficult to be standardize.

Allograft are available in two different preparations: mineralized (fresh-frozen or freeze-dried) and demineralized. Fresh-frozen substrates show high osteoconductivity and appreciable osteoinductivity, while lack osteogenic properties due to osteoprogenitor cells death subsequent to processing and transplantation. Moreover, fresh-frozen grafts are likely to maintain their original biomechanical features. Freeze-dried grafts are poorly osteoinductive and sufficiently osteoconductive, whereas they are characterized by compelling loss of initial biomechanical strength (in particular against bending and torsional forces) due to lyophilisation processing. According to different preparation procedures, freeze-dried allografts are available in three main formulas: cortical, cancellous and particulate. Cortical bone yields structural and mechanical stability while it undergoes slow revascularization and resorption; cancellous and particulate preparations, on the other hand, lack mechanical support but are promptly vascularized and resorbed.

Cortical allografts have been successfully adopted in single-level ACDF and anterior lumbar interbody fusion (ALIF), with fusion rates comparable to those achieved after autograft implantation (8). Cancellous and particulate bone grafts are generally used as autograft extenders in posterior spinal fusion, most notably to treat thoracolumbar deformities (9). However, allograft use has been associated with increased pseudoarthrosis and collapse rates (10), kyphotic deformity and delayed union if compared to autograft, especially in multi-level approaches (11).

Xenograft

Animal allografts use was firstly reported by Maatz and Bauermeister (12), who obtained bone grafts from cows, now commercially purchasable as Kielbone and Surgibone.

Xenograft implantation is associated with relevant issues concerning immunogenicity, low biocompatibility and disease transmission, thus increasing reoperation rates. However, Savolainen et al. have showed no significant differences in fusion rates comparing autograft with xenograft in 250 ACDF (13).

Demineralized Bone Matrix

Demineralized Bone Matrix (DBM) is an allogeneic graft deprived of its mineral phase, which is primarily composed of crystalline calcium hydroxyapatite. Demineralization is achieved through acid extraction processing, which lowers the risk of disease transmission and allows retention of type I collagen, non-collagenous proteins and growth factors.

DBM is thus characterized by osteoinductive and osteoconductive properties, whereas it does not yield any mechanical support because of its amorphousness.

Indeed, DBM is available in different formulas such as putty, gel, flexible sheets or combined with cortical chips. Association of substrates with carrier materials (e.g. glycerol, calcium sulphate powder, hyaluronic acid and gelatine) further improves DBM handling characteristics, albeit occasionally altering its osteoinductive capacity (14).

However, lack of standardization among preparation methods and reliable assessment of growth factors concentration, which both differ widely among manufacturers, makes DBM actual contribution to spinal fusion difficult to be evaluated.

Certain studies have compared the use of DBM alone to DBM with autograft in posterolateral lumbar fusion on animal models. Successful results were reported, even if other authors have demonstrated that DBM alone can not lead to spinal fusion, but may be used as an autograft extender, in order to obtain a more solid spinal arthrodesis (15). Moreover, notable fusion rates were observed subsequent to combination of DBM with a structural carrier, albeit associated with higher rates of pseudoarthrosis and graft collapse if compared to autograft in ACDF (16).

Ceramics

Bioceramics are biocompatible, nontoxic, biodegradable, non-immunogenic and osteoconductive materials currently used for several applications in orthopaedic surgery.

Calcium phosphate ($CaPO_4$) substrates are the most commonly adopted in spine surgery. Synthesis occurs through a thermal reaction termed sintering, in which high temperature (approximately 1000 ºC) leads to formation of a polycrystalline structure. Different processing methods have been engineered in order to fashion products with distinct biomechanical, chemical and structural features. In this regard, porosity should be designed to promote cell infiltration, attachment and neovascularization. The inverse correlation between pore size and biomechanical strength must be taken into account as well.

Calcium phosphate substrates primarily used include synthetic β-TriCalcium Phosphate (β-TCP) and hydroxyapatite, which may be synthetized or coral derived. β-TCP has been extensively utilized as a bone filler: it is highly biocompatible and resorbable, but shows poor structural and impact resistance. On the contrary, hydroxyapatite-based ceramics are characterized by higher crystallinity degrees and, consequently, significant biomechanical strength, while graft resorption is remarkably slow.

Most of bioceramics currently used in spine surgery are composed of β-TCP, hydroxyapatite and occasionally bovine type I collagen, which can bind growth factors such as BMP-2, thus favoring osteogenesis. Indeed, bioceramic grafts lack osteoinductive properties and hence need to be combined with osteogenic substrates (e.g. Bone Marrow Aspirates – BMA) to be possibly considered as a valid substitute for autograft.

Several studies carried on animal models have reported adequate spinal fusion rates using ceramic grafts combined with BMA, while bioceramics alone did not provide any detectable evidence of solid arthrodesis. Likewise, clinical studies have confirmed the efficacy of ceramic substrates associated with BMA; Epstein et al. have evaluated the performance of a porous synthetic ceramic (80% β-TCP and 20% bovine Type I collagen) combined with BMA compared to local bone graft. They showed spinal fusion rates of 96% and 85% in single- and two-level instrumented posterolumbar interbody fusion (PLIF), respectively (17).

Bone Morphogenetic Proteins

Bone Morphogenetic Proteins (BMPs) are cytokines belonging to the Transforming Growth Factor (TGF)-β superfamily that drive bone formation through chemotaxis and differentiation of MSCs into mature osteoblasts, thus leading to osteogenesis. BMPs are classified into four subfamilies according to their functions and analogies. BMP-2 and -7 have been mostly acknowledged for inducing osteogenesis (18).

BMPs are mainly released by osteoblasts, chondrocytes and endothelial cells as active dimers carrying a secretion signal prodomain at the N-terminus and a cysteine-knot motif at the C-terminus (19). Thereupon, BMPs bind to specific receptors (sub classified as Type I and Type II) located at the target cell surface. This interaction induces hetero-oligomerisation of the receptor and activation of the signaling pathway. This leads to subsequent phosphorylation of a group of transcription factors termed SMAD, which activate BMP-mediated gene

expression (20).

To date, BMPs have been used in association with osteoconductive materials to enhance bone graft incorporation and bone neoformation; in addition, these factors seem to counterbalance the inhibitory effect of nicotine on osteoblasts activity. On the other hand, using BMPs may cause potential side effects such as heterotopic bone formation, pseudoarthrosis and inflammation and swelling.

Moreover, isolation and purification processing often results in unacceptable costs. Indeed, BMPs are synthetized using recombinant DNA technology. Urist firstly isolated BMP-2 and BMP-7, which are the two recombinant forms currently approved by the FDA (21). The combination of rhBMP-2 with porous hydroxyapatite based ceramics has been proven effective while performing ACDF, as it yielded appreciable stiffness rates in flexion, extension and lateral bending tests, both in primates and goats (22).

BMPs expression can be induced through gene therapy, which consists of linking the human BMP DNA sequence to a vector (most frequently a virus) capable of transfecting target cells that are further expanded (23). This technique shows significant advantages, such as cost effectiveness, no need for autologous cells harvest or culture and simple methods to assess transduction in vitro. Indeed, a study conducted by Alden et al. on rats injected with BMP-2 gene has reported endochondral bone formation at 12 weeks after injection (24).

Platelet Rich Plasma

Platelet α-granules contain several growth factors, such as TGF-β1, IGF-1, VEGF and PDGF, which are all secreted into the Extracellular Matrix (ECM) upon platelet activation. These soluble factors lead to chemotaxis, mitogenesis and differentiation of osteoprogenitor cells, hence acting as osteoinductive factors (2).

Platelet concentrates may be easily derived from Platelet Rich Plasma (PRP), which is obtained through centrifugation of whole blood taken from the patient before or during surgery (25). Thrombin and/or calcium/chloride are added in order to achieve a gelatinous consistency.

PRP have shown to induce chemotaxis and mitosis of human MSCs in a dose-dependent manner both in vitro and in vivo (2). Walsh et al. assessed the effect of PRP loaded into a resorbable hydroxyapatite calcium carbonate graft or autograft in a sheep model of PLIF. PRP associated with autograft yielded the best outcomes among all the combinations (26).

However, clinical studies have concluded that PRP osteoinductive properties are more difficult to be evaluated in humans. Carreon et al. have recorded a 25% pseudoarthrosis rate in patients treated with PRP and autograft compared to a 17% rate in patients implanted with autograft alone two years after multiple-level spinal fusion surgery (27). Similarly, Castro et al. have reported a 19% lower arthrodesis rate while using PRP in intersomatic fusion surgery (28).

Controversial results in animal and human studies might be due to different reasons, such as prompt gel resorption, low platelet concentration, presence of inhibitory soluble factors and heterogeneity among processing methods.

Bone Marrow Aspirate

Autologous BMA harvested from the iliac crest yields a vast array of MSCs and growth factors, thus enhancing graft incorporation and spinal fusion healing due to its osteoinductive and osteogenic potential.

Even representing less than 1% of the bone marrow cells, MSCs are capable of both self-renewing and differentiating into osteoblasts, chondrocytes, adipocytes and myocytes. Higher MSCs concentration can be obtained through BMA centrifugation in the operating room; the concentrate might be further combined with a PRP gel in order to improve its handling characteristics and prevent MSCs from diffusing away from the injection site. Additionally, MSCs may be either expanded ex vivo through cell culturing or directly embedded onto implantable grafts using a concentrator.

Due to its amorphous nature, BMA needs to be loaded into a solid scaffold, such as ceramic (14). This association has prompted slightly inferior fusion rates if compared to autograft. Moreover, bone marrow concentrate efficacy has been assessed in combination with a PRP gel on cortico-cancellous allogeneic bone graft in cervical and lumbar fusion. This composite material was implanted unilaterally during posterolateral lumbar fusion, while the allograft itself was placed in the contralateral side.

Radiographic evaluation demonstrated higher bone formation at the side treated with the composite substrate (2).

Vertebral Augmentation

Vertebral augmentation, performed through vertebroplasty or kyphoplasty, is a less invasive and motion-sparing procedure used to stabilize a fractured vertebra in order to reduce patient's pain. The aim is to restore mechanical properties of the injured vertebra avoiding the fusion of different motion segments.

Vertebroplasty is a percutaneous technique that consists of an injection of acrylic cement via cannulae into the vertebral body in order to harden the vertebra and give it greater strength and stability. Galibert et al. have used the procedure in 1987 with satisfactory results to treat painful hemangiomas, myelomas and metastatic lesions. Since then, vertebroplasty has been extensively used to augment pedicle screws for spinal instrumentation and to fill cavities (29) resulting from tumor resection in order to reduce the risk of fracture (30). The satisfactory results obtained by these procedures and the resulting pain relief experienced by the patient, led to a widespread use of this technique in painful osteoporotic vertebral fractures (31). Indeed, the procedure has gained acceptance among physicians and is being performed more frequently as an alternative to nonoperative therapy in elderly patients with osteoporotic compression fractures. Several studies have demonstrated good clinical results in terms of pain relief. Indeed, approximately 90% of patients with osteoporotic vertebral fractures (32) and 60-70% of patients with various tumors experienced pain relief after vertebroplasty (33).

While having excellent results on pain, vertebroplasty does not enable fracture reduction and it is associated with a higher rate of cement extrusion. Kyphoplasty is performed to treat the pain caused by an osteoporotic vertebral fracture by recovering the vertebral body height and the sagittal plane loss. The procedure consists of the placement of an inflatable bone tamp into the vertebra in order to restore its height and create a void which can be filled with cement. The results of this technique are similar to vertebroplasty in terms of pain control; with a 50-90% restoration of vertebral height if patients are treated within three months of injury (34).

Polymethylmethacrylate (PMMA) bone cement represents the most frequently injected material in vertebral augmentation. It is supplied as a powder of PMMA with an opacifier and the liquid MMA monomer with hydroquinone and N,N-dimethyl-p-toluidine in a brown glass vial to prevent UV light initiating polymerisation. PMMA is not radiopaque; therefore, radiopacifiers, usually barium suflate, are added to the powder based on the different anatomical district to be used on. In the spine, a much higher opacity is needed to differentiate the cement from the trabeculae. However, opacifiers have various deleterious effects on mechanical properties of PMMA such as reduction of the tensile strength and toughness. Furthermore, granules of contrast agent may initiate a macrophage inflammatory reaction leading to bone reabsorption (35). Embolization due to the initial leakage of cement into vertebral and paravertebral veins during its injection has also been described. Other potential complications related to this substrate are linked to its exothermic reaction as it cures, which may induce necrosis of surrounding tissue. The various disadvantages of using PMMA, which may hinder bone healing in association to its not resorbable nature in vivo have led physicians to consider alternative cements for vertebral augmentation.

Several bioactive and bioresorbable cements are currently under development and may be used for augmentation allowing bone healing, being naturally radiopaque and having a lower or nonexistent exothermic reaction (36). However, their clinical application must still be defined.

Calcium phosphate- and calcium sulfate- based cements are biodegradable and break down in the body at the same rate as new bone is formed. The right formulation, relative amounts and ratio are still being optimised as they may alter the injectability and mechanical properties.

A bioactive cement made of glass-ceramic–reinforced composite material (Orthocomp, Orthovita, Malvern, PA) is biocompatible and able to better restore bone properties showing a lower exothermic reaction than PMMA. Another material used in vertebroplasty is an Experimental Brushite Cement (EBC). It is biocompatible and has a shear-thinning behavior, which is favorable for injection.

Disc Regeneration

Currently, the bases of treatment for damaged IVD are based on symptomatic drugs, physical therapy or surgery, which have limited success. The novel knowledge in IVD pathophysiology, regenerative medicine and tissue engineering holds out the possibility of managing IVD degeneration by normalizing the cell biology of the NP, augmenting the disc space with bioactive hydrogels and stem/progenitor cells, repairing the AF, reestablishing disc height and preventing facet joints overload (37).

Stem cells therapy for disc regeneration

Several authors are currently examining the possibility of using progenitor cells to overcome cell loss within the degenerate disc creating in order to restore the normal proteoglycan content (38). Stem cell transplantation, with or without a hydrogel carrier or porous scaffold, has also been extensively investigated. MSCs from various sources have been shown to restore damaged IVDs in small and large animal models of disc degeneration (39-41). Few reports on the transplantation of stem cells in human patients have been published. Autologous bone marrow derived MSCs were applied together with collagen scaffolds in two patients undergoing decompression surgery for spinal stenosis; symptoms were improved and MRI outcome and disc stability were satisfying after two years (42). In another study, bone marrow MSCs were transplanted into IVDs of ten patients affected by degeneration-related chronic back pain. The treatment led to an improvement of the symptoms for the patients and partial restoration of the disc hydration (43). Despite these promising results, there are drawbacks associated with MSC based therapies. Cells collection, expansion, culture and delivery procedures are invasive, challenging in terms of logistics and therefore cost intensive. Moreover, an extra surgery is required for cell isolation.

Alternatively, endogenous stem cells or progenitor cells (44) induce regeneration as an important part of the natural healing process that occurs as a physiological reaction to tissue injury. This involves the recruitment of endogenous MSCs towards the site of damage in response to chemotactic factors released by the affected cells. Recently, the recruitment of progenitor cells driven by injury has been shown in rat IVDs (45). Moreover, a disc organ culture study demonstrated that degenerative conditions induce the release of factors promoting MSCs recruitment (46). Current research is focused on the investigation on chemoattractant molecules able to recruit MSCs towards the degenerated IVD as well as a chemoattractant delivery system.

Nucleoplasty

Over the past few years there has been a growing interest in research focused on the development of in situ-forming hydrogels for NP regeneration. An ideal hydrogel should not only provide a microenvironment compatible for NP cell survival and progenitor cells engraftment but it should also promote tissue regeneration and have tolerable viscoelastic properties to bear and transfer load applied to the spinal segments. A large variety of hydrogels have been developed and are being tested in valid ex vivo and in vivo models (47-51). Hydrogels have been functionalized with anabolic cytokines or can be used in combination with progenitor cells as a tissue engineering approach.

However, recent studies suggested that NP replacement materials are extruded over time in most of the cases when delivered through the annulus fibrosus (52-55). With these difficulties in mind, we have recently developed an alternative approach to replace the NP through the endplate, named as the transpedicular route (56-58). This new approach has the potential to bring successful nucleoplasty procedures in clinic.

Annulus Fibrosus repair

Novel strategies towards annular repair that would significantly reduce re-herniation rate, IVD degenerative changes and trigger healing processes will drastically improve the quality of life of patients undergoing discectomy. Moreover, NP regenerative strategies are ineffective without a functional AF able to withstand the physiological intradiscal pressure. Therefore, new strategies to augment, repair, or regenerate AF are strongly needed with the attempt to both reduce recurrent disc herniation rate and increase the effectiveness of NP regenerative strategies (59).

Biodegradable synthetic polymers and biopolymers or their combination have been

processed into different scaffolding and membranes to be used in AF repair, some have already a long history in the field of medical devices, while others are newly emerging biomaterials (60-62).

Regenerative therapies, using biomaterials scaffolds loaded with biological factors (e.g. stem cells, growth factors) have been proposed as an additional supplementation to AF self-repair thus increasing longevity of the biological implant (63). Despite promising preliminary results in in vitro and in vivo studies, these technologies are still in their infancy and no solution has yet reached the clinics.

CONCLUSIONS

In the past few years, the fields of biomaterials and regenerative medicine have rapidly grown with significant improvements applied in spine surgery. As the volume of spine surgery continues to increase biotechnologies and biomaterials are aiding surgeons and patients in improving the success rates of spinal procedures with minimal complications.

The necessity for spinal fusion is increasing and major complications such as pseudoarthrosis may impair its results. The continued development of adjuncts to spinal fusion is not static. Several surgical procedures have improved the effectiveness of this technique. Bone substitutes have been developed with the aim to enhance bone healing. However, bone grafts still remain the best option, even if many concerns result from their availability, properties, safety and stockage.

Growth factors and PRP have attracted great interest as adjuvants in spinal fusion due to their biological actions, but opinions on their function and results are still contradictory, except for BMP-2 and BMP-7, which are valid alternatives in spine fusion.

However, spinal fusion compromises spine biomechanics. This clearly stresses the importance of biological approaches to disc repair. Utilizing cells and tissue engineering processes may represent a valid therapeutic approach offering, in the not too distant future, a minimally invasive procedure towards regeneration rather than fusion. IVD regeneration through cell-based therapy could regenerate or at least slow down IVD degeneration, by implanting autologous cells such as MSCs.

Vertebral augmentation, performed through vertebroplasty and kyphoplasty, serve as a bridge between prolonged nonoperative care and open surgical procedures being a highly effective treatment for pain control with minimal risks to patients. However, new biocements and biomaterials need to be developed to better integrate and mimic biomechanical properties of the spinal segment.

Future may bring safer and more efficacious biomaterials and biotechnologies, however, it is crucial to have in-depth understanding of the principles of the different categories and their essential properties as well as their clinical effectiveness. Surgeons need to have the information to carefully and critically evaluate the best treatment.

REFERENCES

1. Di Martino A, Papapietro N, Lanotte A, Russo F, Vadala G, Denaro V. Spondylodiscitis: standards of current treatment. Curr Med Res Opin 2012; 28:689-99.
2. Vadala G, Di Martino A, Tirindelli MC, Denaro L, Denaro V. Use of autologous bone marrow cells concentrate enriched with platelet-rich fibrin on corticocancellous bone allograft for posterolateral multilevel cervical fusion. J Tissue Eng Regen Med 2008; 2:515-20.
3. Gupta A, Kukkar N, Sharif K, Main BJ, Albers CE, El-Amin Iii SF. Bone graft substitutes for spine fusion: A brief review. World J Orthop 2015; 6:449-56.
4. Reid JJ, Johnson JS, Wang JC. Challenges to bone formation in spinal fusion. J Biomech 2011; 44:213-20.
5. Albrektsson T, Johansson C. Osteoinduction, osteoconduction and osseointegration. Eur Spine J 2001; 10(S)2:S96-101.
6. Chau AM, Mobbs RJ. Bone graft substitutes in anterior cervical discectomy and fusion. Eur Spine J 2009; 18:449-64.
7. Sengupta DK, Truumees E, Patel CK, Kazmierczak C, Hughes B, Elders G, Herkowitz HN. Outcome of local bone versus autogenous iliac crest bone graft in the instrumented posterolateral fusion of the lumbar spine. Spine 2006; 31:985-91.
8. Jagannathan J, Shaffrey CI, Oskouian RJ, Dumont

AS, Herrold C, Sansur CA, Jane JA. Radiographic and clinical outcomes following single-level anterior cervical discectomy and allograft fusion without plate placement or cervical collar. J Neurosurg Spine 2008; 8:420-8.
9. Berven S, Tay BK, Kleinstueck FS, Bradford DS. Clinical applications of bone graft substitutes in spine surgery: consideration of mineralized and demineralized preparations and growth factor supplementation. Eur Spine J 2001; 10(2):169-77.
10. Zdeblick TA, Ducker TB. The use of freeze-dried allograft bone for anterior cervical fusions. Spine 1991; 16:726-9.
11. Bishop RC, Moore KA, Hadley MN. Anterior cervical interbody fusion using autogeneic and allogeneic bone graft substrate: a prospective comparative analysis. J Neurosurg 1996; 85:206-10.
12. Bauermeister A, Maatz R. A method of bone maceration; results in animal experiments. J Bone Joint Surg Am 1957; 39(A):153-66.
13. Savolainen S, Usenius JP, Hernesniemi J. Iliac crest versus artificial bone grafts in 250 cervical fusions. Acta Neurochir 1994; 129:54-7.
14. Hsu WK, Nickoli MS, Wang JC, et al. Improving the clinical evidence of bone graft substitute technology in lumbar spine surgery. Global Spine J 2012; 2:239-48.
15. Cammisa FP, Jr., Lowery G, Garfin SR, et al. Two-year fusion rate equivalency between Grafton DBM gel and autograft in posterolateral spine fusion: a prospective controlled trial employing a side-by-side comparison in the same patient. Spine 2004; 29:660-6.
16. Miyazaki M, Tsumura H, Wang JC, Alanay A. An update on bone substitutes for spinal fusion. Eur Spine J 2009; 18:783-99.
17. Epstein NE. A preliminary study of the efficacy of Beta Tricalcium Phosphate as a bone expander for instrumented posterolateral lumbar fusions. J Spinal Disord Tech 2006; 19:424-9.
18. Sanchez-Duffhues G, Hiepen C, Knaus P, Ten Dijke P. Bone morphogenetic protein signaling in bone homeostasis. Bone 2015; S8756-3282(15)00206-9.
19. Mueller TD, Nickel J. Promiscuity and specificity in BMP receptor activation. FEBS Lett 2012; 586:1846-59.
20. Wrana JL, Tran H, Attisano L, Arora K, Childs SR, Massague J, O'Connor MB. Two distinct transmembrane serine/threonine kinases from Drosophila melanogaster form an activin receptor complex. Mol Cell Biol 1994; 14:944-50.
21. Urist MR. Bone: formation by autoinduction. Science 1965; 150:893-9.
22. Spivak JM, Hasharoni A. Use of hydroxyapatite in spine surgery. Eur Spine J 2001; 10(2):197-204.
23. Hubert MG, Vadala G, Sowa G, Studer RK, Kang JD. Gene therapy for the treatment of degenerative disk disease. J Am Acad Orthop Surg 2008; 16:312-9.
24. Alden TD, Pittman DD, Beres EJ, Hankins GR, Kallmes DF, Wisotsky BM, Kerns KM, Helm GA. Percutaneous spinal fusion using bone morphogenetic protein-2 gene therapy. J Neurosurg 1999; 90:109-14.
25. Tarantino R, Donnarumma P, Mancarella C, Rullo M, Ferrazza G, Barrella G, Martini S, Delfini R. Posterolateral arthrodesis in lumbar spine surgery using autologous platelet-rich plasma and cancellous bone substitute: an osteoinductive and osteoconductive effect. Global Spine J 2014; 4:137-42.
26. Walsh WR, Loefler A, Nicklin S, Arm D, Stanford RE, Yu Y, Harris R, Gillies RM. Spinal fusion using an autologous growth factor gel and a porous resorbable ceramic. Eur Spine J 2004; 13:359-66.
27. Carreon LY, Glassman SD, Anekstein Y, Puno RM. Platelet gel (AGF) fails to increase fusion rates in instrumented posterolateral fusions. Spine (Phila Pa 1976) 2005; 30:E243-6.
28. Castro FP, Jr. Role of activated growth factors in lumbar spinal fusions. J Spinal Disord Tech 2004; 17:380-4.
29. Kostuik JP, Errico TJ, Gleason TF. Techniques of internal fixation for degenerative conditions of the lumbar spine. Clin Orthop Relat Res 1986:219-31.
30. Scoville WB, Palmer AH, Samra K, Chong G. The use of acrylic plastic for vertebral replacement or fixation in metastatic disease of the spine. Technical note. J Neurosurg 1967; 27:274-9.
31. Lapras C, Mottolese C, Deruty R, Lapras C, Jr., Remond J, Duquesnel J. Percutaneous injection of methyl-metacrylate in osteoporosis and severe

vertebral osteolysis (Galibert's technic). Ann Chir 1989; 43:371-6.
32. Jensen ME, Evans AJ, Mathis JM, Kallmes DF, Cloft HJ, Dion JE. Percutaneous polymethylmethacrylate vertebroplasty in the treatment of osteoporotic vertebral body compression fractures: technical aspects. AJNR Am J Neuroradiol 1997; 18:1897-904.
33. Cotten A, Duquesnoy B. Vertebroplasty: current data and future potential. Rev Rhum Engl Ed 1997; 64:645-9.
34. Lieberman IH, Dudeney S, Reinhardt MK, Bell G. Initial outcome and efficacy of "kyphoplasty" in the treatment of painful osteoporotic vertebral compression fractures. Spine 2001; 26:1631-8.
35. Sabokbar A, Fujikawa Y, Murray DW, Athanasou NA. Radio-opaque agents in bone cement increase bone resorption. J Bone Joint Surg Br 1997; 79:129-34.
36. Bai B, Jazrawi LM, Kummer FJ, Spivak JM. The use of an injectable, biodegradable calcium phosphate bone substitute for the prophylactic augmentation of osteoporotic vertebrae and the management of vertebral compression fractures. Spine 1999; 24:1521-6.
37. Vadala G, Russo F, Di Martino A, Denaro V. Intervertebral disc regeneration: from the degenerative cascade to molecular therapy and tissue engineering. J Tissue Eng Regen Med 2015; 9:679-90.
38. Sobajima S, Vadala G, Shimer A, Kim JS, Gilbertson LG, Kang JD. Feasibility of a stem cell therapy for intervertebral disc degeneration. Spine J 2008; 8:888-96.
39. Vadala G, Sobajima S, Lee JY, Huard J, Denaro V, Kang JD, Gilbertson LG. In vitro interaction between muscle-derived stem cells and nucleus pulposus cells. Spine J 2008; 8:804-9.
40. Vadala G, Studer RK, Sowa G, Spiezia F, Iucu C, Denaro V, Gilbertson LG, Kang JD. Coculture of bone marrow mesenchymal stem cells and nucleus pulposus cells modulate gene expression profile without cell fusion. Spine (Phila Pa 1976) 2008; 33:870-6.
41. Sakai D, Mochida J, Iwashina T, et al. Regenerative effects of transplanting mesenchymal stem cells embedded in atelocollagen to the degenerated intervertebral disc. Biomaterials 2006; 27:335-45.
42. Yoshikawa T, Ueda Y, Miyazaki K, Koizumi M, Takakura Y. Disc regeneration therapy using marrow mesenchymal cell transplantation: a report of two case studies. Spine 2010; 35:E475-80.
43. Orozco L, Soler R, Morera C, Alberca M, Sanchez A, Garcia-Sancho J. Intervertebral Disc Repair by Autologous Mesenchymal Bone Marrow Cells: A Pilot Study. Transplantation 2011; 92: 822-828.
44. Sakai D, Nakamura Y, Nakai T, et al. Exhaustion of nucleus pulposus progenitor cells with ageing and degeneration of the intervertebral disc. Nat Commun 2012; 3:1264.
45. Sakai D, Nishimura K, Tanaka M, et al. Migration of bone marrow-derived cells for endogenous repair in a new tail-looping disc degeneration model in the mouse: a pilot study. Spine J 2015; 15(6):1356-65.
46. Illien-Junger S, Pattappa G, Peroglio M, et al. Homing of mesenchymal stem cells in induced degenerative intervertebral discs in a whole organ culture system. Spine 2012; 37:1865-73.
47. Collin EC, Grad S, Zeugolis DI, et al. An injectable vehicle for nucleus pulposus cell-based therapy. Biomaterials 2011; 32:2862-70.
48. Gloria A, Borzacchiello A, Causa F, Ambrosio L. Rheological characterization of hyaluronic acid derivatives as injectable materials toward nucleus pulposus regeneration. J Biomater Appl 2012; 26:745-59.
49. Malhotra NR, Han WM, Beckstein J, Cloyd J, Chen W, Elliott DM. An injectable nucleus pulposus implant restores compressive range of motion in the ovine disc. Spine 2012; 37:E1099-105.
50. Peroglio M, Grad S, Mortisen D, Sprecher CM, Illien-Junger S, Alini M, Eglin D. Injectable thermoreversible hyaluronan-based hydrogels for nucleus pulposus cell encapsulation. Eur Spine J 2012; 21(6):S839-49.
51. Silva-Correia J, Zavan B, Vindigni V, Silva TH, Oliveira JM, Abatangelo G, Reis RL. Biocompatibility evaluation of ionic- and photo-crosslinked methacrylated gellan gum hydrogels: in vitro and in vivo study. Adv Healthc Mater 2013; 2:568-75.
52. Di Martino A, Vaccaro AR, Lee JY, Denaro V, Lim

MR. Nucleus pulposus replacement: basic science and indications for clinical use. Spine 2005; 30:S16-22.

53. Omlor GW, Bertram H, Kleinschmidt K, Fischer J, Brohm K, Guehring T, Anton M, Richter W. Methods to monitor distribution and metabolic activity of mesenchymal stem cells following in vivo injection into nucleotomized porcine intervertebral discs. Eur Spine J 2010; 19:601-12.
54. Reitmaier S, Wolfram U, Ignatius A, et al. Hydrogels for nucleus replacement--facing the biomechanical challenge. J Mech Behav Biomed Mater 2012; 14:67-77.
55. Vadala G, Sowa G, Hubert M, Gilbertson LG, Denaro V, Kang JD. Mesenchymal stem cells injection in degenerated intervertebral disc: cell leakage may induce osteophyte formation. J Tissue Eng Regen Med 2012; 6:348-55.
56. Vadala G, De Strobel F, Bernardini M, Denaro L, D'Avella D, Denaro V. The transpedicular approach for the study of intervertebral disc regeneration strategies: in vivo characterization. Eur Spine J 2013; 22(6):972-8.
57. Vadala G, Russo F, Pattappa G, et al. The transpedicular approach as an alternative route for intervertebral disc regeneration. Spine 2013; 38:E319-24.
58. Vadala G, Russo F, Pattappa G, et al. A Nucleotomy Model with Intact Annulus Fibrosus to Test Intervertebral Disc Regeneration Strategies. Tissue Eng Part C Methods 2015; 21(11):1117-24.
59. Vadala G, Mozetic P, Rainer A, Centola M, Loppini M, Trombetta M, Denaro V. Bioactive electrospun scaffold for annulus fibrosus repair and regeneration. Eur Spine J 2012; 21(1):20-26.
60. Chang G, Kim HJ, Vunjak-Novakovic G, Kaplan DL, Kandel R. Enhancing annulus fibrosus tissue formation in porous silk scaffolds. J Biomed Mater Res A 2010; 92:43-51.
61. Nerurkar NL, Baker BM, Sen S, Wible EE, Elliott DM, Mauck RL. Nanofibrous biologic laminates replicate the form and function of the annulus fibrosus. Nat Mater 2009; 8:986-92.
62. Nerurkar NL, Mauck RL, Elliott DM. ISSLS prize winner: integrating theoretical and experimental methods for functional tissue engineering of the annulus fibrosus. Spine 2008; 33:2691-701.
63. Alini M, Roughley PJ, Antoniou J, Stoll T, Aebi M. A biological approach to treating disc degeneration: not for today, but maybe for tomorrow. Eur Spine J 2002; 11(2):215-20.

SILVER COATED PROSTHESIS IN ONCOLOGICAL LIMB SALVAGE SURGERY REDUCE THE INFECTION RATE

F. DONATI[1], G. DI GIACOMO[1], A. ZIRANU[1], S. SPINELLI[1], C. PERISANO[1], M. A. ROSA[2] and G. MACCAURO[1]

[1]Catholic University of the Sacred Heart, Division of Orthopedic and Traumatology, Rome, Italy; [2]Messina University, Division of Orthopedic and Traumatology, Messina, Italy

Silver coatings, used in many surgical devices, have demonstrated good antimicrobial activity and low toxicity. Oncological musculoskeletal surgery have a high risk of infection, so in the last decades, silver-coated mega-prostheses have been introduced and are becoming increasingly widespread. In this study, a retrospective analysis of 158 cases of bone tumors, primary or metastatic, treated between 2005-2015 with wide margins resection and tumor implants reconstruction, was performed. The average age was 59 years (range 11-78 years), the same surgeon with antibiotic prophylaxis according to a standard protocol treated all patients. Silver-coated prostheses were implanted in 58.5% of patients and uncoated tumor prostheses in the remaining 41.5%. Patients were re-evaluated annually and complications were recorded, focusing analysis on infective complications. The average follow-up was 39.7 months: 23.4% of patients died at a median time of 35.3 months after surgery; 18.4% developed complications that required new surgery, of which 12.6% of these were due to infection. Patients treated with silver-coated implants developed early infection in 2.2% of cases against the 10.7% of the patients treated with standard tumor prosthesis. This difference between the two groups was statistically significant. The percentage of late infections occurring from 6 months after surgery was similar in both groups. Silver blood level taken in a sample of patients at different times after surgery, always showed values well below the threshold of toxicity and no patient showed any sign of local or general toxicity secondary to silver. Our study demonstrates that tumor silver-coated implants have a rate of early infection significantly lower than traditional implants, while there were no differences in the rate of late infections as described also in the literature. We recommend the use of silver–coated prosthesis as primary implants for limb salvage surgery in primary or metastatic bone tumors, considering the absence of toxicity and the lower rate of early infection.

Limb salvage surgery following primary or metastatic bone tumors is the treatment of choice in young and old patients with an acceptable life expectancy. Literature shows good survival and functional results with this kind of technique.

Prosthetic reconstruction achieves the best possible level of function in patients who need a wide resection for malignant tumor. The approach to this treatment is more and more frequent thanks to earlier diagnosis and to improved surgical technique and implanted device. However, among the most common possible severe complications to this kind of treatment are: deep infections implant failures, periprosthetic fractures, dislocations and tumor

Key words: megaprosthesis, infection, limb salvage, oncological prosthesis, silver prosthesis coating

Mailing address: Dr. Fabrizio Donati,
Catholic University of the Sacred Heart,
Division of Orthopedic and Traumatology,
Largo Agostino Gemelli 8,
00168 Roma Italy
Tel.:+390630154326
e-mail: fabriziodonati2@hotmail.it

0393-974X (2015)

DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

relapse (1, 2).

Oncological patients are often debilitated by tumor itself, chemotherapy or concomitant illness regarding other organs and the large implants surface is predisposed to bacterial colonization. Rate of infection in this case of treatment ranged from 10-40% with great variability depending on the site of replacement, the age, the resection dimension, the primary malignant tumor involved. Preventing periprosthetic infection is therefore of the utmost importance considering that when there are concomitant poor soft tissue conditions, secondary amputation is sometimes inevitable. Many options were proposed to prevent infections in this kind of surgery. Systemic treatments have a significant role and many intra and perioperative antibiotics prophylaxis have been proposed (3, 4). It is not easy to demonstrate a statistically significant supremacy among antibiotic prophylaxis proposed, but is mandatory to choose one according to the infectious disease specialist indications.

Associated treatment like antibiotic local washing, medicated devices or metals with antimicrobial activity have been studied for years by many authors. Among the metal with antimicrobial activity, silver has garnered much interest due to its excellent antimicrobial activity coupled with low toxicity (5). Different medical devices exploiting the properties of silver are now also available in orthopedics surgery. Silver coated tumor endoprostheses was introduced in medical practice almost 25 years ago with the aim of treating local periprosthetic infections. In literature we can find descriptions of severe early or late general sign of silver toxicity following silver-coated prosthesis implantation in animals or humans. Only few case reports showed local sign of toxicity, like dermal argyria (6).

Considering the above-mentioned results, silver-coated mega-prostheses have recently been proposed as first implant in oncological limb savage surgery in order to prevent onset of infection. In the Orthopedic and Traumatology Division of "A. Gemelli Hospital" (Catholic University of the Sacred Heart, Rome) the use of silver-coated prosthesis as first implant in this kind of surgery began in selected case almost 15 years ago and in the last years involved more and more patients, showing good functional and survival results. To improve functional results and faster recovery our surgical technique involves the use of Trevira Tube® to guarantee soft tissue and capsular reconstruction as described by other authors (7) (Fig. 1).

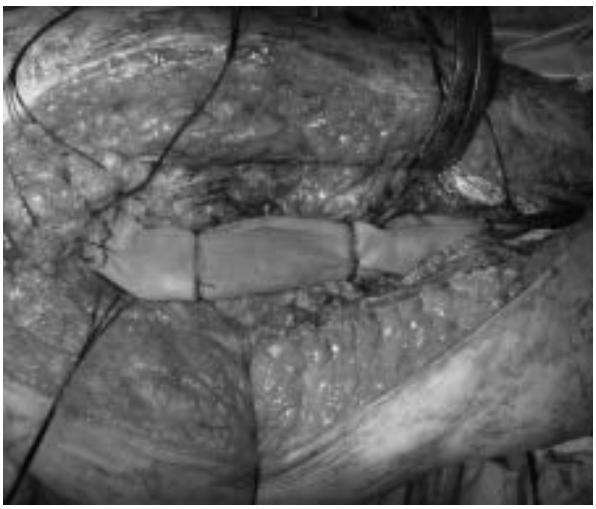


Fig. 1. *Soft tissue reconstruction using Trevira Tube® after proximal femur resection and silver-coated implant.*

The aim of this study was to compare the risk of infection in patients treated with silver-coated prosthesis to patients treated with uncoated tumor implants.

MATERIALS AND METHODS

A retrospective review was performed, analyzing all patients affected by primary or metastatic bone tumors**,** treated with wide margin resection and mega-prostheses reconstruction by the same surgeon between 2005-2015 in the Orthopedic and Traumatology Division of "A. Gemelli Hospital" (Catholic University of the Sacred Heart, Rome) with a minimum of 6-month follow-up.

The overall population counted 158 patients, treated with limb salvage surgery, 71 males and 87 females. Patients who had previous surgery on the tumor site were excluded. The average age was 59.3 years old (range 11-78 years), all patients followed an antibiotic prophylaxis according to a standard protocol. In 62 cases the disease for which the patients had been treated was a primary bone tumor, in the remaining 96 cases it was secondary to a metastatic disease, among them the most common primary tumor was breast, urogenital and thyroid cancer.

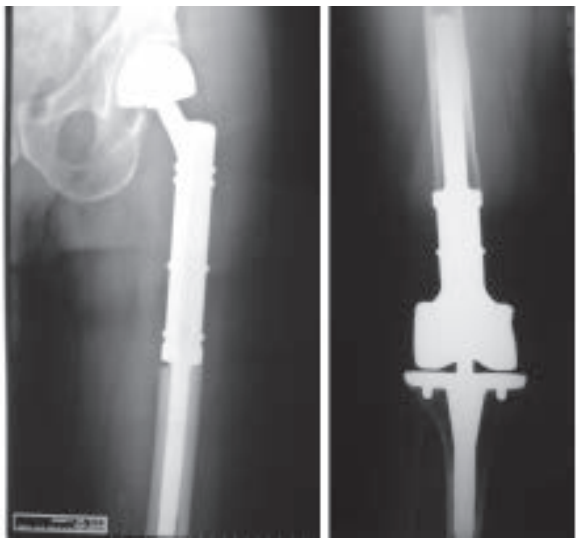


Fig. 2. *Post-operative Xray after proximal and distal femur resection and reconstruction with silver coated modular prosthesis (MUTARS® Implantcast Ltd, Buxtehude, Germany).*

The primary bone tumors were osteosarcoma in 19 patients; in 17 Ewing sarcoma/primitive neuroectodermal tumor; in 14 chondrosarcoma; in 6 malignant fibrous histiocytoma of the bone; in 6 locally advanced stage III giant cell tumor of the bone. Eleven patients had pathologic fractures.

In 93 cases (58.5%) patients were implanted silver-coated modular prostheses (MUTARS® Implantcast Ltd, Buxtehude, Germany) (Fig. 2). Uncoated titan modular tumor prosthesis (MUTARS ® Implantcast Ltd, Buxtehude, Germany) were implanted in the remaining part partients.

In the silver-coated prosthesis group, the anatomical site of resection was proximal femur in 33 cases, distal femur in 19 cases, total femur in 3 cases, proximal tibia in 13 cases, proximal humerus in 18 cases, diaphysis in 1 case, pelvis in 4 cases, distal radio in 1 case and distal tibia in 1 case. In the uncoated tumor prosthesis group the resection site was proximal femur in 25 cases, distal femur in 15 cases, total femur in 1 case, proximal tibia in 10 cases, proximal humerus in 10 cases, diaphysis in 3 cases and distal tibia in 1 case (Table I).

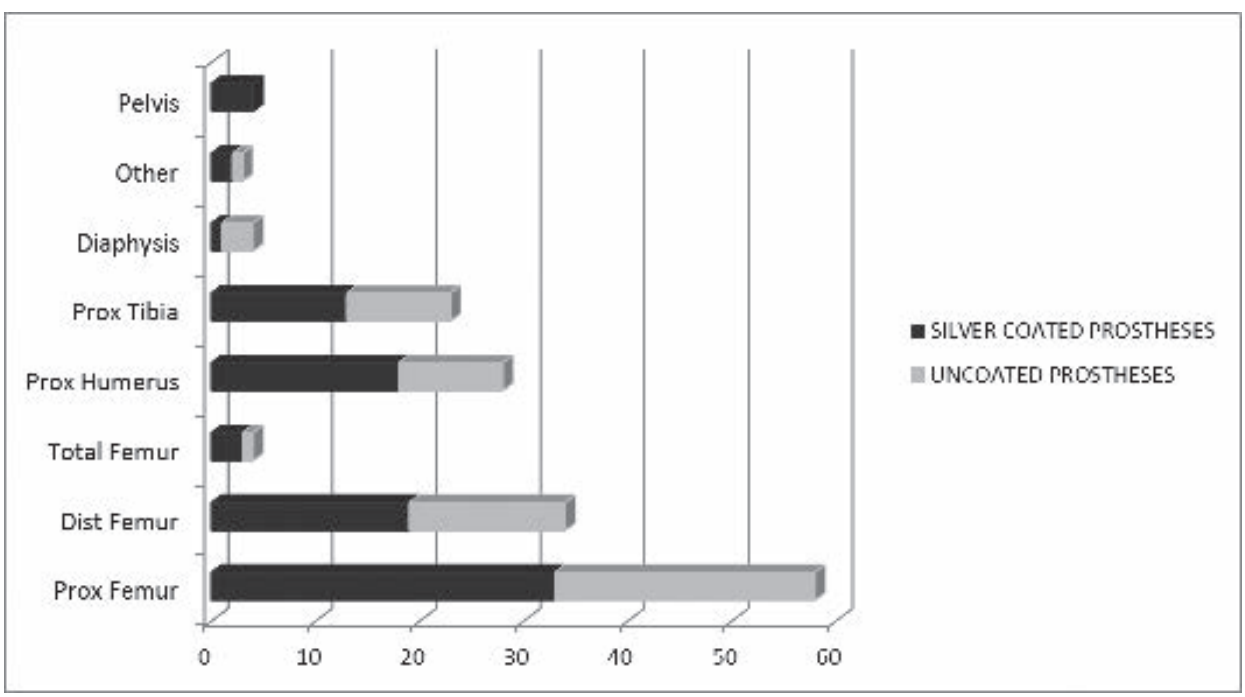


Table I. *Anatomical site distribution in silver-coated prosthesis group and in uncoated tumor prosthesis group.*

All patients received the same antibiotic prophylaxis consisting in 2 mg Cefazolin administered 30 min before surgery followed by 1 mg every 12 h in the three post-operative days. The dimension range of the bone resection was between 10 cm and 70 cm. All patients underwent radio and/or chemotherapy and/or other surgeries as per the indications of the Oncologists of "A. Gemelli Hospital" (Catholic University of the Sacred Heart, Rome). The average time of hospitalization was 11.7 days (min 4 days- max 73 days).

The patients were checked at follow up, 1, 3, 6 and 12 months post-surgery and then once a year. The average follow up was 39.7 months (range 12-114 months). Only the data recorded in the last follow up available were analyzed, considering that 23.4 % of patients died on average 35.3 months post-operative (6 from primary bone tumor and 31 from metastasis). In 18.4% of the cases, severe complications that contemplated the necessity to perform a second surgery for implant failure, dislocation, tumor relapse, or infection, were registered. Among these complications, 20 cases of deep infection were registered. Infection was diagnosed clinically and with laboratory values (C-reactive protein) in the presence of pus and/or if the leukocyte count in fluid aspirated from the periprosthetic cavity was >1,700/10,000 cm3 (8). A statistical study with a multivariate analysis was performed on the cases of infection in order to analyze the risk factors involved in the infection onset.

Early infection was defined as an infection that required second surgery before 6 months from the first surgery. When necessary, the patients underwent antibiotic treatment based on microbiological exams, according to expert infectious disease doctors' indications. The overall average time of infection was 12.4 months after surgery.

The aim of the study was to compare the rate of infections in the group in which silver coated prostheses were implanted versus the group in which standard titan megaprostheses were implanted. The association of gender, age, histology, anatomical site, resection size, time of infection, associated therapy (radio/chemotherapy or other surgery), second surgery for other complications and use of Trevira Tube® were examined. Significance was set at $p<0.05$.

Close clinical surveillance was observed at each follow up to monitor the risk of local or general silver toxicity. Silver blood level was taken in a randomly selected sample of 10 patients for each group, at different times after surgery in order to prevent potential systemic risks.

RESULTS

The two groups were homogeneous in all the characteristics considered. Of the overall population of 158 patients, 20 cases of periprosthetic infection were recorded, corresponding to 12.65% of all cases. Nine cases occurred in the silver-coated prosthesis group that showed a risk of infection of 9.7%. Eleven cases of deep infection were recorded in the group in which uncoated titan prostheses were implanted (16%). Overall, the different risk level of infection between the two groups was not statistically significant but silver-coated prostheses showed a slightly lower risk.

The multivariate analysis showed no statistically significant difference between the two groups for gender, age, histology, anatomical site, resection size, associated therapy, second surgery for other complications and use of Trevira Tube®. A resection bigger than 30 cm, concomitant complications and use of Trevira Tube® seem to be negative prognostic factors but the small number of cases involved did not allow the study to obtain a statistical significance.

The only significant differences between the two groups were related to the onset of infection. In the group treated with silver-coated implants, 2 cases (2.2%) underwent second surgery for infection before 6 months after surgery, while in the other group early infection occurred in 7 cases (10.7%). The comparison between the two groups showed evident statistical significance ($p<0.05$). Late infection occurred in 7 patients treated with silver

Table II. *Early infection occurring before 6 months after the first surgery was significantly lower in silver-coated prosthesis group. No difference were detected between the two groups for late infection risk.*

	Early infections	Late infections	Total
Silver coated tumor prostheses	**2 (2.2%)**	**7 (7,5%)**	**9 (9,7%)**
Titan uncoated tumor prostheses	**7 (10.7%)**	**4 (6,2%)**	**11 (16%)**

coated prostheses (7.5%) and in 4 patients treated with uncoated prostheses (6.2%). No statistically significant difference for late infection risk was detected in the two groups (Table II).

Patients never showed local or general signs of secondary toxicity to silver exposition at the considered times. Wider resection and silver blood level values taken on 10 patients in each group were also well below the threshold of toxicity in every sample.

DISCUSSION

Periprosthetic infection of megaprostheses continues to be a frequent and major complication in orthopedic oncology. The results of our study show that the development of prosthetic silver coatings can significant lower early infection rates compared to the uncoated titan tumor prostheses. Currently, many practices limit periprosthetic infection through modern operating rooms with laminar airflow, systemic antibiotic treatment and routine screening for multi-drug-resistant bacteria that become common cause of infection. The production of an effective inhibition are through antimicrobial silver-coated surface may be useful to prevent the adherence of organisms (allowing leaching of silver off the coated surface) (9, 10), not only on the coated surface but also for a variety of host-derived adhesins, such as fibronectin, fibrinogen, fibrin and laminin, that exist within the biofilm layer (11, 12). .

Many authors demonstrated that the silver coating of a prosthesis can decrease the infection rate due to the release of silver ions that produce an inhibition zone and the resulting coated prostheses are more likely infection-resistant in vivo (13, 14, 15). In this study, the silver coating also showed a protective role, especially in early infection but the protective effect is no longer present in late infections. This is likely related to wear of the silver coating, which apparently occurs on average around 2 years after implantation. In our experience, silver coating wear appears evident even in visual macroscopic analysis of different explanted prostheses. The theory is that the silver coating loses effect by that time due to physiological mechanical erosion although further *in vivo* studies are required in order to obtain a scientific confirmation and to establish how long silver-coated can be considered protective after implantation.

It remains unclear if silver coating could lead to other types of infection (e.g., time of infection, virulence) or to more frequent soft-tissue infections. In literature, leukocyte scintigraphy demonstrated increased uptake, particularly in the superficial soft tissues. In our view, this can be explained by active free silver ions, binding to proteins, become inactivated (silver ions could build complexes with serum albumin) (16). In these areas, the silver coating is unable to develop an adjuvant effect as demonstrated by Schierholz et al. (17). Free silver ions may precipitate in albumin-containing environments (e.g., hematoma), leading to concentrations that are too low for bactericidal effects to be achieved. In our study, rate of infection was not significantly related to these kinds of complications or to the dimension of the resection, probably because of the small number considered. However, we feel that we can confirm the theory that the surgeon must avoid hematoma and poor muscle coverage of the prosthesis, which results in superficial wound healing problems and bacterial colonization; for these reasons we routinely use from one to three wound drainages up to 2 days after surgery.

Patients with a relatively large amount of released silver ions show a faster decrease in the inflammatory marker CRP, even if is unclear whether the concentration of released silver ions in the immediate surroundings of the prosthesis has an influence on the clinical course.

In comparison to other metals with antimicrobial activity (like copper, cadmium, mercury) (5, 18), silver has shown good antimicrobial activity and low toxicity. Silver toxicity has been reported to occur at serum levels as low as 0.3 mg/mL and manifests as argyria, leukopenia and alterations in renal, hepatic, and neural tissues (19, 20). Thus, it is prudent to incorporate silver on the surfaces of the prostheses in adequate concentrations to reduce bacterial adherence but not high enough to cause systemic toxicity. In our study, no patient ever showed local or general sign of toxicity secondary to silver ions exposition and in literature only few case of dermal argyria are described after this kind of surgery.

Lastly, the economic factor has to be considered. The silver-coated megaprosthesis is admittedly 5-7% more expensive than the uncoated prosthesis

(14). However the significant decreased period of hospitalization and the decreased revision surgeries rate must be taken in consideration as relevant cost factors, as demonstrated by other authors (21, 22).

CONCLUSIONS

Our data suggest that considering the lack of sign of toxicity and the statistically significant lower rate of early infection, silver coated megaprosthesis are safe and useful to improve the clinical outcome in limb salvage surgery. Therefore, we recommend the use of silver-coated prosthesis as primary implants in all limb salvage surgery in primary or metastatic bone tumors. A complete eradication of risk of infection with silver coating is unrealistic therefore, in absence of any statistical evidence we suggest avoiding hematoma and wound-healing disturbances by achieving sufficient muscle coverage of the prosthesis. In the future, more studies are necessary to confirm these results and to study for how long a time after implantation, silver coating has a protective role *in vivo*.

REFERENCES

1. Palumbo BT, Henderson ER, Groundland JS, Cheong D, Pala E, Letson GD, Ruggieri P. Advances in segmental endoprosthetic reconstruction for extremity tumors: a review of contemporary designs and techniques. Cancer Control 2011; 18(3):160-70.
2. Puchner SE, Kutscha-Lissberg P, Kaider A, et al. Outcome after Reconstruction of the Proximal Tibia - Complications and Competing Risk Analysis. PLoS One 2015; 10(8): e0135736.
3. Racano A, Pazionis T, Farrokhyar F, Deheshi B, Ghert M. High infection rate outcomes in long-bone tumor surgery with endoprosthetic reconstruction in adults: a systematic review. Clin Orthop Relat Res 2013; 471(6):2017-27.
4. Hardes J, Gebert C, Schwappach A, et al. Characteristics and outcome of infections associated with tumor endoprostheses. Arch Orthop Trauma Surg 2006; 126:289-96.
5. Tobin EJ, Bambauer R. Silver coating of dialysis catheters to reduce bacterial colonization and infection. Ther Apher Dial 2003; 7:504-9.
6. Hardes J, Ahrens H, Gebert C, et al. Lack of toxicological side effects in silver-coated megaprostheses in humans. Biomaterials 2007; 28:2869-75.
7. Gosheger G, Hillmann A, Lindner N, Rödl R, Hoffmann C, Bürger H, Winkelmann W. Soft tissue reconstruction of megaprostheses using a trevira tube. Clin Orthop Relat Res 2001; (393):264-71.
8. Trampuz A, Hanssen AD, Osmon DR, et al. Synovial fluid leukocyte count and differential for the diagnosis of prosthetic knee infection. Am J Med 2004; 117:556-62.
9. C. von Eiff, G. Peters, C. Heilmann. Pathogenesis of infections due to coagulase-negative staphylococci. Lancet Infect Dis 2002; 2(11):677-85.
10. E. Sheehan, J. McKenna, K. J. Mulhall, P. Marks, D. McCormack. Adhesion of Staphylococcus to orthopaedic metals, an in vivo study. J Orthop Res 2004 Jan; 22(1):39-43.
11. Hermann M, Vaudaux PE, Pittet D, et al. Fibronectin, fibrinogen, and laminin act as mediators of adherence of clinical staphylococcal isolates to foreign material. J Infect Dis 1988; 158:693-710.
12. Vaudaux P, Pittet D, Haeberli A, et al. Host factors selectively increase staphylococcal adherence on inserted catheters: a role for fibronectin and fibrinogen or fibrin. J Infect Dis 1989; 160:865-75.
13. H. Ahrens, G. Gosheger, A. Streitburger, C. Gebert, J. Hardes. Antimikrobielle Silberbeschichtung von Tumorprothesen. Der Onkologe 2006; 12:145-51.
14. G. Gosheger, J. Hardes, H. Ahrens, et al. Silver-coated megaendoprostheses in a rabbit model-an analysis of the infection rate and toxicological side effects. Biomaterials 2004; 25(24):5547-56.
15. Hardes J, von Eiff C, Streitbuerger A, Balke M, Budny T, Henrichs MP, Hauschild G, Ahrens H. Reduction of periprosthetic infection with silver-coated megaprostheses in patients with bone sarcoma. J Surg Oncol 2010; 101(5):389-95.
16. N. Shahabadi, M. Maghsudi, Z. Ahmadipour. Study on the interaction of silver(I) complex with bovine serum albumin by spectroscopic techniques. Spectrochim Acta A Mol Biomol Spectrosc 2012; 92:184-8.
17. Schierholz JM1, Lucas LJ, Rump A, Pulverer G. Efficacy of silver-coated medical devices. J Hosp Infect 1998; 40(4):257-62.

18. Kim TN, Feng QL, Kim JO, Wu J, Wang H, Chen GC, Cui FZ. Antimicrobial effects of metal ions (Ag+, Cu2+, Zn2+) in hydroxyapatite. J Mater Sci Mater Med 1989; 9:129-34.
19. Tweden K, Cameron JD, Razzouk A, Holmberg W, Kelly S. Biocompatibility of silver-modified polyesters for antimicrobial protection of prosthetic valves. J Heart Valve Dis 1997; 6:553-61.
20. Wan AT, Conyers RAJ, Coombs CJ, Masterton JP. Determination of silver in blood, urine, and tissues of volunteers and burn patients. Clin Chem 1991; 37:1683-7.
21. von Eiff C, Proctor RA, Peters G. Coagulase-negative staphylococci: pathogens have major role in nosocomial infections. Postgrad Med 2001; 110(4):63-4, 69-70, 73-6.
22. Herrmann M, Peters G. Catheter-Associated Infections Caused By Coagulase-Negative Staphylococci: Clinical and Biological Aspects. Marcel Dekker, New York, NY, USA, 1997.