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CONTENTS

Nutritional assessment of an Italian population of elderly hip fracture patients: a comparison between sexes and types of fractures

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Malnutrition is highly prevalent in elderly patients with hip fractures (HF) (intracapsular and extracapsular). Many factors influence the patterns of HF, but the role of nutrition is not yet clear. In this investigation, an analysis of the body compositions of geriatric patients with HF was conducted, to identify differences in the nutritional status between male and female patients with intra- and extra-capsular HF. The nutritional assessment of patients was performed using three different techniques: anthropometrics measurement, plicometry, and bioimpedance analysis. The most prevalent type of fracture in females was the extracapsular type, while the intracapsular type is more common in males. Males showed a lower BMI, fat percentage and a greater length of hospital stay (LOS). Patients with intracapsular fractures are more malnourished compared with patients with extracapsular fractures. Males with HF have a higher prevalence of intracapsular fractures compared to women and stayed in hospital longer

The number of HF in the elderly is on the rise due to the aging of the population (1). HF are one of the most common and severe fragile fractures, and they are an important sentinel event in elderly patients. They reveal that patients' health condition are compromised (1). In these patients, osteoporosis, the leading cause of HF, is not an isolated condition, but can be the consequence of a group of related comorbidities such as malnutrition, sarcopenia and cachexia (2). Older patients with HF are often frail and sick, in addition to having complex medical problems and comorbidities (1). Elderly people with HF are often malnourished or at risk of becoming malnourished at the time of the fracture and during the hospital stay (1), with relevant consequences regarding clinical and socio-economic implications, given the higher complication rates and prolonged hospitalization, resulting in higher costs for hospitals and health insurance companies (3). To address this problem, identification of malnourished individuals and those at nutritional risk is the essential first step of a comprehensive nutrition care program. HF patients often do not achieve their required nutritional intake (1). Poor nutrition is a risk factor for poor wound and fracture healing, and can be detrimental to health and rehabilitation (1). Large cohort studies showed a close association between malnutrition and increased complication rate, length of hospital stay and costs (4).

We chose to use BMI, anthropometrics measurements with plicometry, and BIA together to have a more comprehensive picture of hip fracture patients. We hypothesized that there would be

Key words: hip fracture, elderly, malnutrition, anthropometrics, BIA, plicometry

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differences in the considered outcomes (BMI, body fat percentage, days before surgery and length of hospital stay) between the subgroups.

MATERIAL AND METHODS

Study design

This is an observational study on malnutrition in HF patients. Participants were recruited during their admission for surgery on a HF at our hospital, from March 2018 to July 2018. Inclusion criteria were, 65 years and older; the patients were alive for the entire duration of the admission. Patients with pacemakers or implanted defibrillators were excluded from BIA (as BIA can interfere with implanted electrical devices), but they were included in the study and analyzed only by anthropometric measurement and plicometry. Other exclusion criterias were known cancer-related pathologic fractures, malignancies, previous or ongoing radiotherapy or chemotherapy.

MATERIALS AND METHODS

There was no need to obtain informed consent as the collection of data was part of the routine evaluation of the patients' status. Participants were included consecutively, and data were collected within 48 hours from the patients' admission.

Bioimpedance Analysis (BIA)

Participants were measured in the supine position by single frequency tetrapolar BIA (Body fat Analyzer GIMA, Gessate, Italy). Bia was performed following ESPEN guidelines (5)non-invasive, relatively inexpensive and can be performed in almost any subject because it is portable. Part II of these ESPEN guidelines reports results for fat-free mass (FFM. BIA measured total body water (TBW), extra cellular water (ECW), intra cellular water (ICW), body cellular mass (BCM), fat free mass (FFM), fat mass (FM), muscle mass (MM), resting metabolic rate (BMR).

Anthropometric measurements

The anthropometric parameters were evaluated using the following measurements: height, body weight, body mass index (BMI), waist circumference, waist-to-hip ratio, waist-to-height ratio and mid upper-arm circumference.

Height was estimated using demi-span. The height measurements estimated from the demi-span were made with a flexible non-elastic measuring tape (Measuring Tape SECA 203, Hamburg, Germany. The left arm was used when measurements could not be performed on the right one. Height was calculated with Bassey's equations: Height (cm) for men = (1.40*demi-span) +57.8. Height (cm) for women = (1.35*demispan) +60.1. BMI was calculated as weight/(height)² (kg/m2).

Individuals were considered malnourished if their BMI was less than 20, normal from 20 to 24.9, overweight if ≥ 25, and obese if ≥30 (6)BMI was obtained for 3110 (1663 males and 1447 females. Body measurements were always taken on the right side of the body. However, some measurements were taken on the left side of the body because of casts, open wounds, or amputation.

To measure the mid upper-arm, abdominal (waist), thigh and calf circumferences the Anthropometry Procedures Manual of the National Health and Nutrition Examination Survey (NHANES) was followed.

Plicometry

The thickness of the skinfolds was measured with a pincer-type caliper (Harpenden® Skinfold Caliper, Bologna, Italy). The procedures were performed following the NHANES manual. The anatomic sites for skinfold measurements included biceps, triceps, subscapular, abdominal, supra-iliac and upper thigh.

Durnin and Womersley's equations were used to obtain the Body Density Value which was then converted to Body fat percentage using Siri Equation (7)Siri or Brozek, based on the two compartment model, provides a more accurate conversion of body density (BD by the Skinfold Body Assessment Software provided with the Harpenden Caliper. We calculated FFM and FM indices to compare our results with published reference data and to classify patients with defined cut-off value (8). The FFM and FM indices are equivalent concepts to the BMI, as shown in the following definition:

Mathematically, BMI (kg/m²) = FFMI (kg/m²) + FMI (kg/m²).

Patients were classified as malnourished if their FMI was under the 25th percentile value, and "overfat" rather that overweight if the FMI was over the 75th percentile of the reference data of the Italian population (8).

Statistics

All statistical analyses were conducted using Microsoft Excel. Categorical variables are presented as a frequency (percentage), and continuous variables are presented as a mean ± standard deviation. The Chi-square test was used to compare categorical variables, and the Mann-Whitney U test and Student's t-test were used to compare continuous variables. For all tests, a value of $P < 0.05$ indicates statistical significance.

RESULTS

A total of 131 eligible patients was admitted to the study. Of these, two died before any measurement could be taken, and eight patients were not compliant enough to be measured. Data are therefore presented on 121 patients, 31 male (25.6%) and 90 females (74.4%); 83 patients with a 31.A (extracapsular) fracture (68.6%) and 38 patients with a 31.B (intracapsular) fracture (31.4%) (Table I.)

Of these 121 patients, six patients had a pacemaker and could not undergo BIA, but only skinfold and anthropometric measurement. Analysis of variance, comparing the surgical delay and the LOS in 3 groups (underweight, normal weight and overweight for BMI; malnourished, normal and overfat for fat percentage) of patients showed no significant difference between the groups. Logistic regression showed no relation between age and the site of fracture. Patients were classified as malnourished, normal and overweight according to the percentage of fat mass measured by plicometry. We found no differences in distribution among sub-groups (Table II).

There were substantial differences between the four groups. The most prevalent type of fracture was the 31.A type (68.6%), but the 31.B type has a higher prevalence in the males (48.4%). Male patients showed a lower BMI, fat percentage and a greater LOS compared to female patients (Table III). Patients with a 31.B fracture showed a lower BMI (23.4 kg/m²) and fat percentage (27.9% with BIA and 26.6% with plicometry) compared to patients with a 31.A fracture, but they did not differ in surgical delay and LOS (Table III).

DISCUSSION

There is no definitive consensus on the diagnosis of malnutrition in HF patients, which explains the variability and the difficulty in management (9). Most published studies on malnutrition in HF include small samples, or exclude some types of patients so they cannot represent all HF patients (9).

Studies which use anthropometric criteria

Table I. *Cohort data.*

Variable	Males N=31/121 (25.6%)	Females N=90/121 (74.4%)	31.A N=83/121 (68.6%)	31.B N=38/121 (31.4%)	Total N=121
Age	82.1±7.7	83.2±7.1	82.8±7.3	82.5±7.1	83.1±7.3
Weight (kg)	63.1±10.3	62.2±11.9	63.5±11.0	60.2±12.3	62.4±11.5
Height (m)	1.63±0.01	1.56±0.05	1.56±0.05	1.6±0.07	1.57±0.06
BMI	24.2±3.1	25.7±4.6	26.4±4.2	23.5±4.1	25.4±4.4
Body fat % (plicometry)	22.8±5.5	31.7±9.7	30.8±9.8	26.6±8.7	29.4±9.6
Body fat % (BIA)	23.6±4.4	32.8±9.1	32.1±0.1	27.9±8.6	30.7±9.1
Water %	49.1±14.3	46.4±10.1	46.2±10.6	48.9±12.4	47.1±11.2
Days before surgery	2.2±2.4	1.6±1.0	1.6±0.8	2.2±2.3	1.8±1.5
LOS (days)	12.5±4.7	10.8±2.7	11.0±2.8	11.6±4.4	11.2±3.4

Data are expressed as a mean ± standard deviation.

(primarily BMI and anthropometric measurements) show a prevalence of malnutrition between 5 and 52% (9). Body composition is a key component of health, and its measurement is increasingly considered valuable in clinical practice (9). Several techniques are available, varying in complexity and ease of use, and each one makes assumptions that may affect its suitability for different conditions. A single technique is unlikely to be optimal in all circumstances (10).

All body composition methodologies are based on assumptions regarding the density of body tissues, concentrations of water and electrolytes, and/or biological interrelationships between body components and body tissues and their distributions among healthy individuals (10). There is no single universally recommended method for body composition assessment, but each modality has benefits and drawbacks (10). For these reasons we used multiple methods of measurement and, to our knowledge, this is the first study using three different techniques to classify the nutritional state of elderly patients with HF.

Malnutrition negatively influences recovery after fracture, it increases complications, mortality and

Table II. *Differences in nutritional status among sub-groups.*

Variable	Underweight	Normal	Overweight
Females	43.3%	28.9%	27.8%
Males	61.3%	29.0%	9.7%
31.A	43.2%	29.6%	27.1%
31.B	57.5%	27.5%	15.0%
Total	47.9%	28.9%	23.1%

Data are expressed as a mean ± standard deviation.

Table III. *Comparison between sexes and types of fractures.*

Variables	Males (31)	Females (90)	p	31A (81)	31B (40)	p	Statistic test
Age (years)	83.2±7.1	82.1±7.7	0.71	82.8±7.3	81.5±7.9	0.31	Mann-Whitney
Fracture type (31B n.)(%)	15(48.4 %)	25(27.8%)	0.035	16(51.6 %)	15(48.4%)	0.035	Chi Square
BMI (kg/m²)	23.7±2.9	25.7±4.6	0.017	26.4±4.2	23.4±4.1	0.002	Student t test
Surgical delay (days)	2.2±2.4	1.6±1.0	0.72	1.6±0.8	2.2±2.2	0.41	Mann-Whitney
LOS (days)	12.5±4.7	10.8±2.7	0.044	11.0±2.8	11.6±4.4	0.82	Mann-Whitney
Body Fat % with BIA	23.6±4.4	32.8±9.1	0.008	32.1±9.1	27.9±8.6	0.017	Mann-Whitney
Body Fat % with Plicometry	22.8±5.5	31.7±9.7	0.02	30.8±9.8	26.6±8.7	0.016	Mann-Whitney

Data are expressed as a mean ± standard deviation.

healthcare spending (4). Nutritional intervention aids the prevention of complications in geriatric patients with HF (4). The observed variability in the parameters of nutritional status in hip fracture patients could result from the lack of a universal consensus as to the best measure to diagnose protein-energy malnutrition (11).

Despite this, the observed trend is uniform: malnourished older people are at a greater risk of fracture, and the prevalence of malnutrition is high in geriatric patients admitted with a HF. Patients with intracapsular fractures usually have low BMI, while patients with trochanteric fractures tend to have a higher BMI (11).

Based on anatomy, HF can be divided into intracapsular fractures (femoral neck fractures) and extracapsular fractures (basicervical, intertrochanteric and subtrochanteric fractures). These two types of fracture have different pathogeneses and require different treatment strategies. Multiple factors, including the geometry of the hip, areal bone mineral density (BMD), age, pre-injury function status, history of fragile fractures, severe hip osteoarthritis, affect the patterns of HF, although few studies address this specific issue (12). A low BMI is a well-known risk factor for HF and a recognized risk factor for a poor outcome following a HF (13).

Also, malnutrition contributes significantly to euthyroid sick syndrome (14, 15) and frailty syndrome (16). Frail subjects have lower lean body mass, appendicular skeletal mass and lower skeletal muscle index than normal and overweight subjects (16). Overall, frailty is significantly associated with the decline of physical function and changes of body composition, which may be a consequence of loss of bone and muscle mass (16). A strong association between frailty and lower BMD, in both the lumbar spine and hips of older adults, has been identified. A significant health hazard of frailty is falls and related fragility fractures (16). Frail subjects are more likely to fall, and to have osteoporosis, as well as sarcopenia and HF (16).

Therefore, a low BMI is a well-established risk factor for osteopenia and sarcopenia, and malnutrition can lead to a decline in BMD (17). Old age is a risk factor for death within the first year after a HF, as is, for unknown reasons, male gender (18). The mortality rate in men is almost twice that in women during the first year after the fracture (18). The longer mean hospital stay in our male patients is in agreement with the literature that report morbidity and mortality following a HF to be greater in men compared to women (18). The mortality in men one year after the fracture is 18% and only 8% in women who fracture at the same age (19). Also, men are more likely to experience a postoperative complication, and represent a subpopulation of HF patients at increased risk of death who may need closer monitoring during the perioperative period (20).

It is not clear yet why men sustain a higher rate of intracapsular fractures. Some hypotheses are related to the differences in bone architecture and strength between males and females (21). Some factors shown to contribute to the strength of the femoral neck include bone size geometry, cortical thickness, and cortical bone density (21). Sex-related differences in osteoporosis-related fracture rates have often been attributed to higher bone mineral density (BMD) in men than women. While it is plausible that differences in bone density may, in part, explain sex-related differences in fracture rates, it is also possible that differences in bone size and geometry may contribute to differences in both bone density and fracture rates (22).

BMI may affect bone geometry of the femoral neck. The two types of nutrient deficiency influence femoral shape in different ways: a type I deficiency occurring before skeletal maturity can lead to relative shortening of the true femoral neck and consequent mechanical femoral neck protection (23). Type 1 nutrients (such as iron, calcium, zinc and vitamins) are those required for specific metabolic functions in the body and they are stored in the body. With a deficient diet the person continues to grow normally (24). Type 2 nutrients (such as protein, zinc, magnesium, potassium, sodium, phosphorus and sulphur) are necessary in every metabolic pathway and are important for maintaining weight. Deficiencies of type I nutrients gives rise to biochemical abnormalities without any necessary anthropometric changes, whereas deficiencies of type II nutrients give rise to anthropometric abnormalities without specific or diagnostic biochemical changes

(24). Therefore, in general a low BMI is associated to a type II nutrient deficiency, but will not indicate whether there are co-existing type I deficiencies (23).

The present study has several limitations. We lack data on comorbidities and complications, most of which are treated by general practitioners without hospital contacts. After acknowledging this problem in our setting, an orthogeriatrician was hired to achieve an integrated care model. The integration and participation of the GP in the patient evaluation process would make data more reliable. We do not have data on mortality during the hospital stay because the family of the only two deceased patients did not give consent to the collection of data. These patients were in critical conditions and both died the day after the admission. We lack a control group of healthy elderly to compare the result we obtained in the population of hip fracture patients.

Another limitation is that the patients' history was often not accurate enough to determine the exact trauma mechanism: many patients had dementia or were not able to describe the dynamics of the fall. One strength of this study is the inclusion of patients with cognitive dysfunction. Cognitive dysfunction is often an exclusion criterion in prospective studies of HF patients. The other strength of the study is the use of validated instruments and protocols, for anthropometry, plicometry and BIA.

In conclusion, male HF patients have longer mean hospital stay, with lower body fat percentage compared to women. These factors negatively affect the prognosis and the outcomes. Moreover, the prevalence of different kind of fractures varies between males and female patients and more studies are needed to elucidate the causes of this difference.

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Conventional progesterone receptors (PR) B and PRA are expressed by human chondrocytes and can be involved in the pathogenesis of osteoarthritis

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To evaluate the expression, location and role of progesterone receptors (PRs) A and B in human chondrocytic cell lines, Western blotting, real time PCR analyses, transmission electron microscopy and immunogold assays were performed. By transfection and co-transfection assays, the influence of progesterone (OHPg) on estrogen receptor alpha (ERα) promoter activity was investigated. MTT and pAKT documented OHPg effects on chondrocytes survival. The PR-B and PR-A were both observed in human chondrocytes. The PR-B was evidenced both in the nucleus and in the cytosol of the cells. OHPg, through PR-B, induced ERα expression by acting at the ER promoter level affecting chondrocytes survival. We reported for the first time the expression of PRs in human chondrocytes. Interestingly, we described a novel mechanism via progesterone induction of ERα, which may explain, at least in part, the dramatic rise in OA prevalence among postmenopausal women.

Osteoarthritis (OA), a worldwide condition, is strongly associated with aging and is the most common type of arthritis. Although important advances have been made in understanding the physiopathological processes of OA, the mechanisms that could be targeted to control its progression have yet to be determined. Because several degenerative joint diseases have a high female-to-male preponderance, a regulatory role of specific sex hormones has been implicated in controlling metabolism of these tissues (1-3).

It is well known that progesterone (OHPg) is an essential regulator of several female reproductive events (4, 5). However, no data on the role of OHPg on chondrocytes physiology are reported. The biological actions of OHPg are generally mediated via intracellular OHPg receptors (PRs) belonging to the superfamily of transcription factors (6, 7). Human PR is expressed from a single gene as two proteins, PR-A (94 kDa) and PR-B (116 kDa), with distinct functional activities. Generally, PR-B it is a stronger transcriptional activator than PR-A (8). Since an increasing OA prevalence has been reported during post-menopause and andropause period, there is a possibility that the deficiency of progesterone may play a role in its pathogenesis. Recently, the expression of estrogen receptors α and β (ER α, ER β) and androgen receptor (AR) in immortalized C-28/I2 and T/C-28a2 chondrocytes and in human articular cartilage was demonstrated (9), affirming that chondrocytes may represent target cells for sex steroids. Their importance in the chondrocyte's physiology emerged from a recent study on the C-28/

Keywords: osteoarthritis, progesterone receptors, chondrocytes, steroids

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I2 and T/C-28a2 chondrocytes cell lines (10).

Herein, we evidenced for the first time that the PRs are expressed in human chondrocytes, having a role on cell survival. Furthermore, we investigated a possible mechanism through which OHPg and its receptor PR-B may influence cartilage's physiology.

MATERIALS AND METHODS

Chemicals

17-Hydroxyprogesterone (OHPg), Bovine Serum Albumin (BSA) protein standard, Laemmli sample buffer, prestained molecular weight markers, sodium bicarbonate, sodium lactate, sodium pyruvate, dimethylsulfoxide (DMSO), Earle's balanced salt solution (EBSS), Ab Colloidal gold conjugated anti-rabbit IgG secondary Ab and all other chemicals were purchased from Sigma Chemical (Milan, Italy). Acrylamide bisacrylamide was from Labtek Eurobio (Milan, Italy), Triton X-100 were from Farmitalia Carlo Erba (Milan, Italy). Enhanced chemiluminescence (ECL) Plus Western blotting detection system, Hybond™ ECL™, Hepes Sodium Salt were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Antibodies against human PR, β-actin, peroxidase-coupled anti-mouse secondary Ab, non-immune rabbit serum, were from Santa Cruz Biotechnology (Milan, Italy).

Cell cultures

Immortalized C-28/I2 and T/C-28a4 human chondrocytes, which are widely used to study chondrocyte growth and differentiation, were evaluated in the current study (11). The cells were plated and grown for 12, 24, or 48 hours (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. Some samples grown in MC, starved for 24h and were treated for 24h with increasing OHPg from 1 nM to 100 nM. Other sets of experiments included cells grown in MC and then starved for 12, 24, 48 or 72h in serum-free medium (-S). Monolayer-cultured chondrocytes between passages 1 and 7 were used.

Immunoblotting

Western blotting from cell lysate was performed as previously described (12). The concentration of protein was determined using a protein assay kit (Bio-Rad, Milan, Italy, 500-0006) at 595 nm. The specificity of the Ab was performed using immune rabbit serum instead the primary Ab.

RNA extraction, cDNA synthesis, and real time RT-PCR

Total cellular RNA was extracted from cells using Total RNA Isolation System kit (Promega, Madison, WI) as indicated by the manufacturer's recommendations. PCRs were performed in the ABI Prism 7000 Sequence DetectionSystem (Applied Biosystems, Foster City, CA 94404, USA) in a total volume of 30 µl reaction mixture, using the SYBR Green Universal PCR Master Mix 2 x (Applied Biosystems) Negative controls contained water instead of first-strand cDNA. Each sample was normalized based on its 18S ribosomal RNA content. The real time RT- PCR was performed as previously described (13). Primer pairs as follow: PR-B forward, 5′-TAGTGAGGGGGCAGTGGAAC-3′, and reverse, 5′-AGGAGGGGGTTTCGGGAATA-3′ (14). T47D breast cancer cells were used as positive control (15).

Immunogold labelling of PRs

Immunogold labelling for PRs was performed as previously reported (16). The sections were subsequently examined using a Zeiss EM 900 transmission electron microscope. To assess the specificity of the immunolabeling, a negative control was performed in those chondrocytes' sections incubated with the non-immune rabbit serum instead the primary Ab.

Cell survival analysis

Cells (3×10^4 cells/mL) were plated in 24-well plates and grown in the different experimental conditions. The 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium (MTT) (2 mg/mL) (Sigma Aldrich, Milan, Italy) assay was performed as follows as previously described (17). 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.

Plasmids

The following plasmids were used: the wild-type

human ERα (HEGO) (18), the ERα gene promoter (19) the full-length PR-B consisting of the full-length PR-B cDNA fused with the SV40 early promoter and expressed in the pSG5 vector (a gift from Dr. D. Picard, University of Genève, Switzerland).

Transfections and luciferase assays

Cells (1×10^5) were plated into 24-well dishes with 500 µl of regular growth medium per well the day before transfection. The medium was replaced with that lacking serum on the day of transfection, which was done using Fugene 6 reagent as recommended by the manufacturer (Roche Diagnostics, Milan, Italy) with a mixture containing 0.5 µg of a full length ERα gene promoter expression vector previously described (14), and 10 nM OHPg significantly increased the activity of ERα gene promoter construct. Moreover, we co-trasfected the above-mentioned construct with a plasmid over expressing PR-B and treated with OHPg.

Renilla reniformis luciferase expression vector pRL-Tk (Promega, Madison, WI) was used for transfection efficiency. The luciferase activity was measured using Dual Luciferase Assay System (Promega, Madison, WI).

Statistical analysis

Western blot analysis, TEM with immunogold analysis, Realt-Time PCR, Transfections and luciferase assays were performed at least in three independent experiments. Statistical analysis was performed using ANOVA followed by Newman-Keuls testing to determine the differences in the means. $p<0.05$ was considered statistically significant. All statistical analyses were performed using the SPSS software.

RESULTS

Western blotting analysis of PRs in human chondrocytes

The presence of PRs proteins in human chondrocytes was investigated by Western blot (Fig. 1A) using a rabbit polyclonal Ab raised against the C-terminal region of the human PR. In replicated samples two immunoreactive bands, at the molecular mass values of 116 and 94 kDa corresponding to the PR-B and PR-A respectively, were observed both in C-28/I2 and T/C-28a4 cells. As positive controls, T47D human breast cancer cells, which expressed both isoforms (13), were used. The bands were not detected by non-immune rabbit serum (Fig. 1B) indicating that the Ab is specific for the PRs.

PR-B mRNA and Immunogold localization in human chondrocytes

To identify and quantify mRNA levels of PR-B in human chondrocytes, real-time PCR was performed. As shown in Fig. 2A, PR-B mRNA was expressed at appreciable levels in C-28/I2 and T/C-28a4 cell lines (Fig. 2A).

The PRs may induce genomic and non-genomic effects also based on their location. Ultrastructural immunogold analysis sections revealed that the conventional PRs were associated both with the nucleus and the cytosol (Fig. 2A, B). Simultaneous negative control experiments with the normal rabbit serum instead of primary Ab did not show any labelling (Fig. 2B, C).

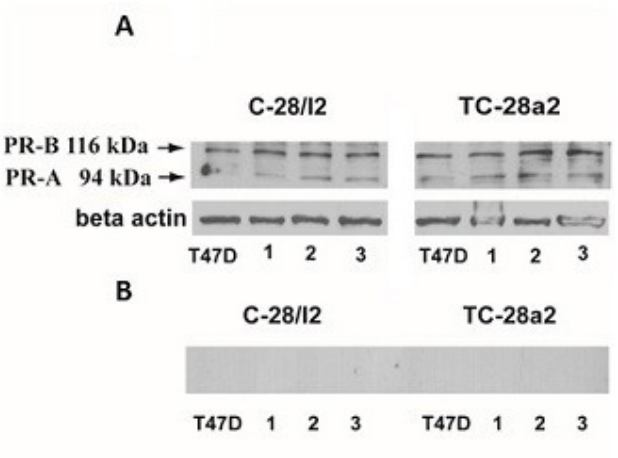

FIG. 1. *PRs proteins are expressed in human chondrocytes. **A**): Western blotting analysis of PR-B and PR-A in C-28/I2 and T/C-28a2 cells grown in MC. T47-D human breast cancer cells are used as positive control of PRs expression. β-actin was used as a loading control. The autoradiographs show the results of one representative experiment repeated at least three times; **B**): The filter was stripped and blotted using a non-immune rabbit serum and bands were not detected.*

OHPg induces cell survival in C-28/I2 and T/C-28a4 human chondrocyte cell lines and OHPg through PR-B induces ERα expression in C-28/I2 cells by a genomic mechanism

Increasing OHPg concentrations induced human chondrocytes cell survival. As shown in Fig. 3A, after 24 hours of treatment, OHPg 10 nM induced a significant increase of pAKT.

Next in a time course assay with OHPg 10 nM cell survival was evaluated performing MTT assay. In both cell lines, cellular viability decreased at 12 and 24 hours compared to time 0, but it significantly increased at 72 hours with 10 nM OHPg in C-28/I2 (Fig. 3B).

On the basis of the ER importance in the cartilage physiology and since there is considerable biological evidence for a cross talk between ER and PR in different cell types (14, 20), we evaluated the ability of OHPg to modulate the expression of the ERα and ERβ. As shown in Fig. 3C, 10 nM and 100 nM OHPg were able to increase ERα expression in C-28/I2 cells, while ERβ exhibited unvaried content (Fig. 3D).

By transient transfection assays in C-28/I2 cells, using a full length ERα gene promoter expression vector previously described (14), 10 nM OHPg significantly increased the activity of ERα gene promoter construct (Fig. 3E). Moreover, we found that PR-B over expression greatly enhanced the OHPg-induced effect on the ERα promoter activity reaching about 200% of induction over untreated, suggesting the specific involvement PR-B in the modulation of ERα promoter activity.

DISCUSSION

The incidence of OA increases with age in both men and women. Premenopausal women have a lower incidence for OA than postmenopausal women or aged-matched men. The descriptive

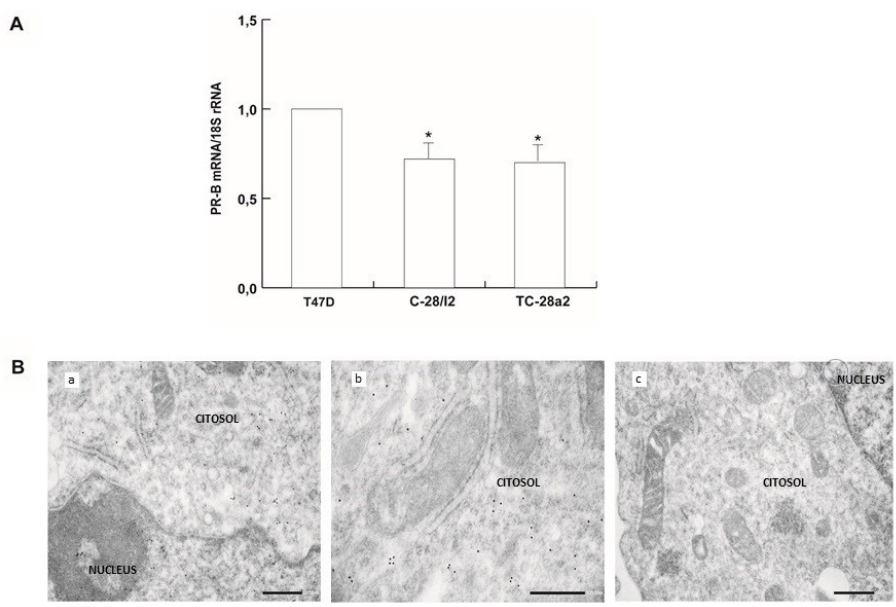

FIG. 2. *PR-B mRNA and Immunogold localization in human chondrocytes. **A**): Real-time PCR assay of PR-B mRNA expression in T47-D, C-28/I2 and T/C-28a2 cells grown in MC, as indicated. 18S rRNA was determined as control. Columns are the mean of three independent experiments each in triplicate; bars, SD; * p ≤0.05 vs T47-D cells; **B**): Immunogold localization of PRs in human chondrocytes. (a-b) TEM analysis was conducted in C-28/I2 cells grown in MC. The figure shows the results of one representative out of three independent experiments; **C**): Negative control experiments were conducted using the normal rabbit serum instead of primary Ab. (scale bar = 500 nm).*

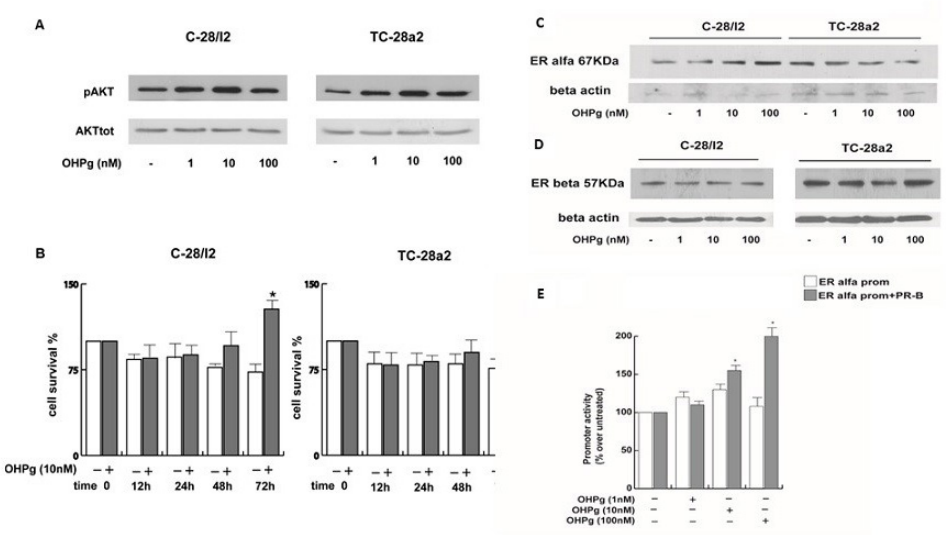

FIG. 3. *OHPg sustains cell survival in C-28/I2 and T/C-28a2 human chondrocyte cell lines, induces ERα expression and ERα gene promoter activity in C-28/I2 cell lines.* ***A****): Western blotting analysis of p-AKT and AKT in C-28/I2 and T/C-28a2 cells treated with different concentrations of OHPg as indicated;* ***B****): MTT assay. Cells were seeded in MC and after 24 hrs were counted (time 0) left untreated or treated with 10nM OHPg for different times as indicated. Results are represented as % of time 0. Columns are the mean of three independent experiments each in triplicate; bars, SD; * p ≤0.05 vs time 0;* ***C****): Western blotting analysis of ERα and D) ERα in C-28/I2 and T/C-28a2 cells treated with different concentrations of OHPg as indicated. β-actin was used as a loading control;* ***E****): By transient transfection assays in C-28/I2 cells, using a full length ERα gene promoter expression vector previously described, 10 nM OHPg significantly increased the activity of ERα gene promoter construct. The construct was co-trasfected with a plasmid over expressing PR-B and treated with OHPg. Columns are mean of three independent experiments and expressed as % over untreated; bars SD; * p ≤0.05 vs vehicle.*

epidemiology of the OA resembles other diseases in which sex hormones play a role. These observations have led to the hypothesis that sex hormones, especially estrogen, may be involved in the etiology of OA, however a possible role for OHPg has never been addressed. The present study was designed to identify, to localize the PR isoforms in human chondrocytes and to examine a possible role for OHPg/PR in chondrocytes biology and/or in the OA physiopathology. By deepening the role of OHPg/PR in chondrocyte's physiology, we evaluated a possible link with the ERs.

First, we identified PRs in human chondrocytes at different levels: PR protein and PR messenger RNA expression. By Western blot analyses both PR-B and PR-A were present. From our data, the PR-B is more expressed with respect the PR-A suggesting that in the chondrocyte physiology the OHPg effects are mainly mediated by the PR-B. Depending on the location of PRs, genomic and non-genomic effects can be induced; in the current study, ultrastructural immunogold analysis revealed that the PRs were present both in the nucleus and in the cytosol.

In somatic cells, downstream signaling proteins potentially involved in mediating OHPg activity include Akt, a key kinase in survival of the cells (21, 22). In our study, increasing OHPg concentrations induced the Akt phosphorylation. Since our data indicated that 10nM OHPg was the more effective dose to induce AKT we performed a time course prolonged up to 72 h in both chondrocytes cell lines, by MTT assays, corroborating a role of OHPg in the chondrocyte's survival.

Several studies indicated that OHPg via

PR-B regulate ERα expression in breast cancer cells at the level of gene transcription (14, 23). Regarding OA, estrogens affect articular cartilage metabolism directly via estrogen receptors (ER) α and β in chondrocytes (9). Our data showed that the expression of ERα, and not ERβ, increased upon OHPg treatment. Interestingly, the OHPg-induced regulation of ERα expression was due to a direct action on the ERα promoter. The PR-B over-expression greatly increased the ERα promoter activation.

In conclusion, we reported for the first time that the PRs are expressed in human chondrocytes strengthening the role of sex steroid in the chondrocyte's biology. We discovered a new molecular mechanism that may help to explain the cartilage-protective effects of progesterone. A potentially novel mechanism via progesterone induction of estrogen receptors, may explain, at least in part, the dramatic rise in OA prevalence among postmenopausal women.

ACKNOWLEDGEMENTS

SA, OG and GG conceived this study and had full access to all the data. OG, GG and DAR provided study materials and patients. SP, FDA and MS performed most of the experiments and acquired the data. SA and FDA took responsibility for the accuracy of the data analysis and wrote the manuscript. MS and SP performed the statistical analysis. DAR performed a critical revision of the manuscript to verify important intellectual content. SA takes responsibility for the integrity of the work. All authors read and approved the final manuscript. This work was supported by MIUR Ex 60 % - 2018.

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Prevention and treatment of peri-prosthetic joint infection using surgical wound irrigation

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Over the past decade, the incidence of revision arthroplasty due to infection has increased substantially, often resulting in multiple surgical interventions with variable success rates and poor clinical outcome. Intraoperative wound irrigation has been proposed to reduce bacterial bioburden and contamination, but currently there is no widely accepted recommendation for the use of topical antiseptics, whether as separate molecules or as a mixed solution. We reviewed studies regarding the use of intraoperative topical antiseptics, their security profile and efficacy in preventing and treating infections of orthopedic implants and introduced a possible combination that may prove valuable in the future.

Peri-prosthetic joint infection (PJI) remains a challenge for orthopedic surgeons, is associated with a high mortality rate, ranging from 2.7%-18.0%, and results in prolonged hospital stay, increased patients disability and finally elevated costs for healthcare system (1, 2). Extensive debridement and wound irrigation in association with antibiotic therapy are the current state of the art in the treatment of acute and chronic infections in total joint replacements (2, 3, 4). Even though antibiotic therapy plays an adjuvant role to mechanical biofilm removal (5), there is still an high number of patients with a recurrence of infection after surgery (3). For these reasons, implementing the efficacy of wound irrigation could lead to reducing the failure rate.

Despite the large amount of literature dealing with the outcomes of irrigation and debridement, little is known about which one is the best solution, its composition and its concentration, resulting in a clinical practice based on experience rather than evidence. Different solutions using various antiseptics alone or in association with the aim to reduce local bacterial load are described. Even though it has been proven that combining antimicrobials could enhance their activities (6) and could help overcome acquired microbial resistance related to the topical use of antibiotics (4, 7), the efficacy and type of irrigation of deep and superficial tissues is inconclusive. Povidone iodine appears to be efficient and safe, so its local use is advocated for by the World Health Organization (WHO) (8) and Center for Disease Control [CDC (G1)] (1), as stated in ICM 2018, while solutions containing hydrogen peroxide and chlorhexidine are also widely used in daily activity due to their activity against a broad spectrum of organisms (7). The purpose of this review is to evaluate the efficacy of different antiseptics such as povidone-Iodine, hydrogen peroxide and chlorhexidine solutions used alone or mixed, to prevent and treat PJIs.

MATERIALS AND METHODS

In order to identify relevant papers dealing with the different antiseptic solutions to prevent and treat PJI, we carried out a research of English literature using the

Key words: wound irrigation; TKA; THA; hydrogen peroxide, povidone iodine, chlorhexidine

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MEDLINE database with the following search items: 'wound irrigation AND joint AND chlorhexidine', 'wound irrigation AND joint AND Povidone Iodine', 'wound irrigation AND joint AND hydrogen peroxide', 'orthopaedic OR orthopedic AND wound irrigation', 'orthopaedic OR orthopedic AND wound lavage, 'orthopaedic OR orthopedic AND hydrogen peroxide', 'orthopaedic OR orthopedic AND Povidone Iodine' and 'orthopaedic OR orthopedic AND chlorhexidine'. Additional papers were identified by checking the references. A total of 591 papers were extracted. Two authors (VdM and ML) independently reviewed each abstract. Once a paper was identified as likely to be included, full-text versions were obtained. Conflict about the inclusion of a paper was resolved by further evaluation which was undertaken by the senior author (GB). Therefore, 15 papers were finally included in the review (Fig. 1).

RESULTS

Hydrogen peroxide

Compared to other antimicrobials, Hydrogen Peroxide (HP) has the advantages of producing non-toxic by-products and can potentially help mechanical debridement with its effervescence. HP's mechanism of action is influenced by its concentrations, in fact, 3% of HP showed a higher bactericidal and antibiofilm activity than various antiseptic solutions. Furthermore, diluted lavage with HP has shown excellent results in clinical settings in reducing infection rate in primary and revision arthroplasty, especially when mixed with povidone iodine (9). Indeed, more recently Chen et al. showed that HP lavage before wound closure in multi-segmental lumbar spine surgery can effectively reduce blood loss and SSI rate, through an indirect mechanism correlated to its hemostatic properties (Table I) (10).

However, the local use of HP raises some concerns. Among its side effects, the damage on the patients' tissues and prosthetic's materials, toxicity towards keratinocytes, fibroblasts and its local oxidative effect and its ability to induce air embolism. This complication has been long known and has been related to the use of HP in closed environments such as intramedullary canal and arthroscopy (11). The interaction of HP and various implant materials has also been widely investigated. Exposure of Titanium alloy and Hydroxyapatite to HP could lead to slight degradation of both; in contrast the effervescence of HP could help to liberate bone trabeculae from fat, blood and debris, therefore allowing a deeper penetration of cement and stronger bone-cement bond.

Chlorhexidine

Chlorhexidine gluconate (CHG) has been approved by FDA as a routine intraoperative solution to prevent and treat infections. Its mechanism of action consists in a disruption of osmotic balance with potassium and phosphorus leakage, leading the cell to death, while at higher concentrations, CHG can cause coagulation of intracellular molecules (12). Solutions containing CHG have demonstrated to have a broad spectrum of activity and are highly effective against a wide variety of organisms responsible for PJI, like Staphylococcus aureus (including methicillin-resistant Staphylococcus aureus -MRSA-) and coagulase-negative Staphylococci. It also demonstrates activity against gram-negative bacteria, fungi and to a lesser extent, mycobacteria (13) (Table I).

However, since most uses of the CHG are limited to intact skin, very few adverse events have been reported. The most frequent adverse reaction to CHG is related to serious hypersensitivity reactions. When applied in contact with mucous membranes, CHG solution has shown to be cytotoxic to human fibroblasts, this effect is time and dose dependent, but the potential adverse local and systemic effects from intra-articular use of CHG remain largely uninvestigated.

Povidone-iodine

Povidone-iodine (PI) is a largely used antiseptic solution consisting of 99% polyvinylpyrrolidone (PVP) and 1% iodine. The association with PVP adds a hydrophilic behavior to deploy the free iodine directly to the cell surface. Once iodine enters the cell, it acts as an oxidant to various components of the cytoplasmic membrane and inactivates several cytoplasmatic molecules, hence damaging irreparably the bacterial, viral and fungal cellular integrity (14). The most common commercially

available formulation is 100 g/L (10%), which is both bactericidal and cytotoxic (15). The clinical efficacy of PI solutions is well described in different papers (Table I). Brown et al (16) investigated in 2012 the correlation between betadine irrigation and the onset of infections after TJA. They showed a significant reduction in the PJI rate after primary Total Hip Arthroplasty and Total Knee Arthroplasty (THA & TKA), soaking the wound for 3 minutes using a 0.35% PI solution and then washing with isotonic NaCl pulsatile lavage.

Since 2012, subsequently, irrigation of the wound using dilute PI before closure following TJA has been widely adopted by many orthopedic surgeons and entered several protocols (17, 18), including spinal surgery (14, 19). In 2016 and 2017, two major guidelines issued by the WHO and CDC recommended this practice (8, 1). In lower limb prosthetics like TKA and THA, several works and reviews stated the efficacy of PI lavage in prevention of PJI (20). Calkins et al. (21), conducted a randomized controlled trial of 457 patients undergoing TKA or THA, and compared the 234 who received wound irrigation with standard saline solution with 223 who received the PI-lavage-protocol refined by Brown et al, finding that 8 infections appeared in the saline group and 1 in the betadine group (16). In 2018, diluted PI-lavage was the only recommended wound irrigation solution by the International Meeting Consensus in Philadelphia (ICM) (7**).**

Still, consensus throughout scientific community is lacking on whether the lavage performed before wound closure following TJA is sure to reduce the risk of infection, due to variations in the concentrations used across the various studies on the subject and unsatisfying results (22). Hernandez et al. analyzed 11,738 cases (23), finding no significant differences between the groups that underwent PI or saline wound irrigation in Primary TKA and THA. Hart et al. (24) encountered similar results with Revision TKA and THA 2884-cases study. The potential negative effects of dilute povidone-iodine lavage are debated: researchers have studied the possible influence on the bone-cement interface, and deleterious effects on articular cartilage. Chondrotoxicity correlated positively with the length of exposure to the diluted PI solution, regardless of the concentration when applied for longer than 1 min. This may prove relevant in Hip Resurfacing, Unicondylar Knee Prosthesis, or retention of patellar button during TKA. Clearly further studies are required on the matter, but given the minimal economic burden and the potential benefit of at least reducing the rate of a terrible complication like PJI, PI-wound irrigation may prove a solid yet incomplete option to the operative orthopedic surgeon.

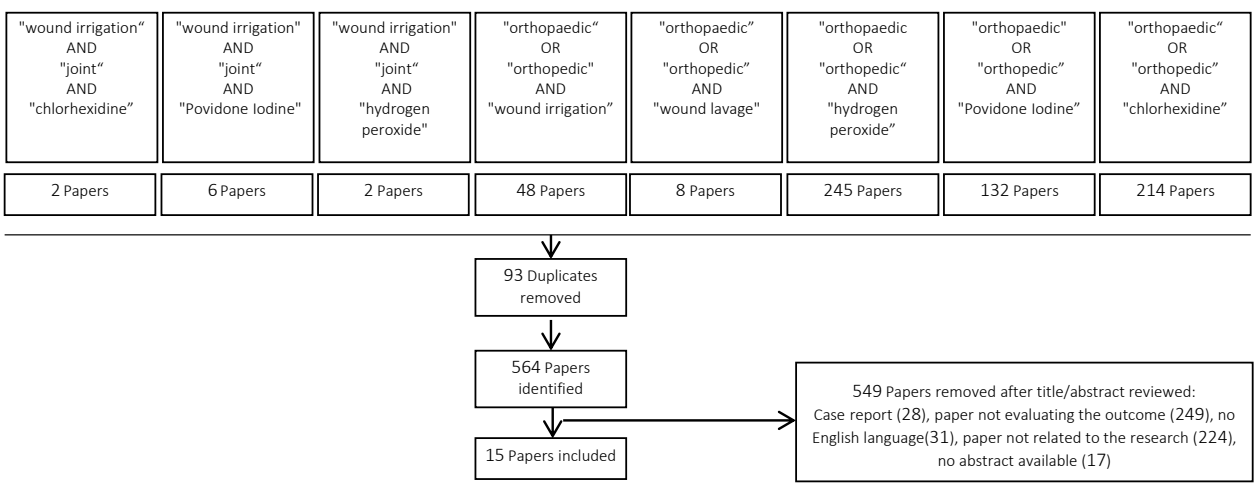

Fig. 1. *Flow chart of randomized controlled trials and cohort studies identified for the review.*

Our proposal

We proposed a new irrigation solution which consists in 500 ml of 0.9% saline sterile solution added with 18 ml of 10% aqueous PI and 125 ml of 3% HP, mixed using a 0.2 µm bacterial filter to avoid contamination. In surgical setting, we use this irrigation solution both in primary and revision total joint replacement. Our protocol is made up of four steps (Fig. 2). First, the wound is soaked for at least 3 minutes using half of previously described irrigation solution just before implantation of the prosthesis, taking care of closing medullary canals using compressed gauze to minimize the risk of pulmonary embolism.

This is followed by pulsatile lavage irrigation with normal saline. After implantation of prosthetic components (stem and cup in THA or femoral and tibial component in TKA) and before the implantation of mobile parts (liner for TKA and femoral head in THA), the wound is further irrigated with the remaining solution for at least 3 minutes followed by pulsatile lavage irrigation. In case of spacer implantation, after synovectomy, implant removal and accurate debridement, the wound is irrigated using half of the prepared solution followed by pulsatile lavage with saline. We used the remaining part of irrigation solution after spacer implantation and just before wound closure, followed again by pulsatile lavage with normal saline.

DISCUSSION

PJIs represent a severe complication deserving multiple surgical procedures and prolonged antibiotic therapies with a high failure rate (3). Extensive debridement and wound irrigation in association with antibiotic therapy are the current state of the art in the treatment of acutely and chronically infected total joint replacements (2). Deep irrigation can potentially reduce the number of microbes in the surgical wound and is particularly useful in prevention and treatment of PJIs. Indeed, diluted betadine lavage prior to skin closure decreased acute infection rates from 0.97% to 0.15% (16).

Acknowledging that the quality of evidence is low, the CDC (1) and the WHO (8) agreed that surgical

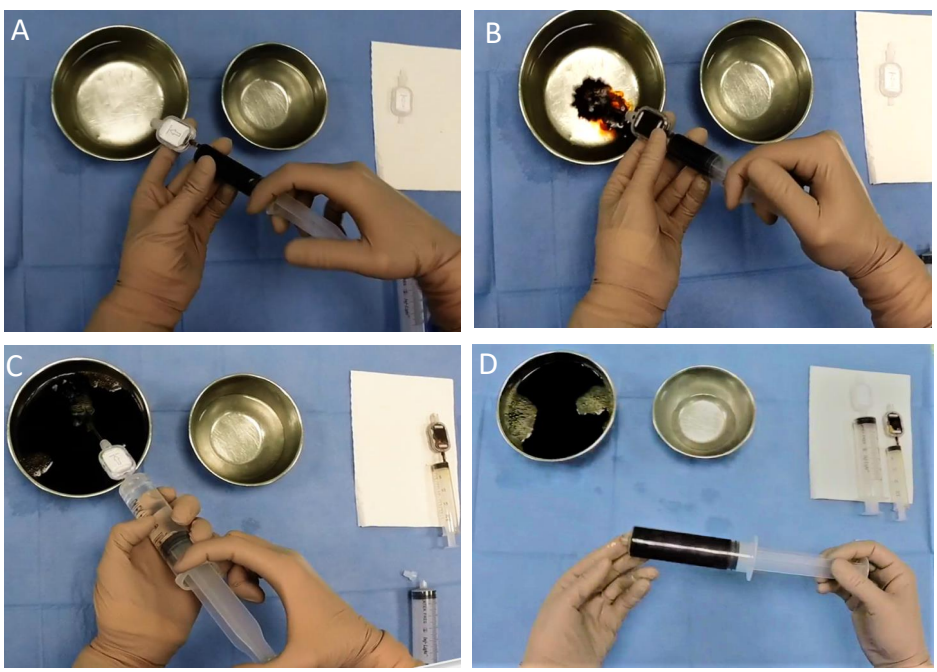

Fig. 2. *Preparation of the irrigation solution.* ***A)****: 500 ml of 0.9% saline sterile solution are poured in a sterile basin;* ***B)****: 18 mL of 10% aqueous PI was added with a 0.2 µm filter;* ***C)****: 125mL of 3% HP was then mixed to previous solution;* ***D)****: the final solution consisting of 643 mL of a brownish and slightly foamy solution.*

Table I. *Summary of clinical studies included in the search.* ***PI****=Povidone Iodine;* ***HP****= Hydrogen Peroxide;* ***CGH****= Chlorexidine Gluconate.*

Paper	N° of patient	Solution	Concentration (v%)	Soaking Time	Type of Study	Site	Rate of infection (%n)
George et al. 2015[22]	N/A	HP followed by PI	1,5% 10%	Not disclosed	Surgical tecnique	Infected TKA	N/A
Chen et al. 2020[10]	2626	HP	3%	30 sec	Retrospective	Spine	1,40%
Sierra et al. 2009[11]	8699	HP	3% swabs	Variable 1-4 min	Retrospective	THA	N/A
Haddad et al. 2015[20]	102	HP + PI	Not disclosed	Not disclosed	Retrospective	TKA, Revision TKA	0% 7%
Ulivieri et al. 2011[26]	950	HP + PI	0,19% 6,25%	1 min	Prospective	Spine	0%
Kosashvili et al. 2011[9]	887	PI followed by HP then Bacitracin	5% 1% 50.000	Not disclosed	Prospective	Revision THA	2,14%
Frisch et al. 2017[13]	664 386	PI vs CGH	<2% 0.05%	2 min 1 min	Retrospective	THA	1,4% – 2%
Slullitel et al. 2019[15]	2890	PI	0.45% 0.2%-0.35%	3 min 1-3 min	Retrospective	TKA, THA, Hip resurfacing (HR)	0,80%
Brown et al. 2012[16]	1862	PI	0.35%	3 min	Retrospective	TKA, THA	0,15%
Hart et al. 2019[24]	2884	PI	0.35%	3 min	Retrospective	THA, TKA	3 months: 3,45% 12 months: 5,9%
Fleischman et al 2018[17]	10949	PI	Not disclosed	Not disclosed	Retrospective	TKA	0,24%
Hernandez et al. 2019[23]	11738	PI	0.25%	3 min	Retrospective	TKA, THA	3 months:0,55% 12 months: 0,695%
Calkins et al. 2019[21]	457	PI	0.35%	3 min	Retrospective	TKA, THA	0,40%
Cheng et al. 2005[19]	414	PI	0.35	3 min	Retrospective	Spine	0,00%
Chang et al. 2006[14]	244	PI	0.35%	3 min	Retrospective	Spine	0,00%

incisions should be irrigated with aqueous iodophor solutions with the aim to prevent superficial and deep SSIs. Furthermore, irrigation solutions, including saline or antiseptics such as sterile dilute povidone iodine are recommended in the treatment of acute and chronic periprosthetic joint infections. Despite the large amount of literature dealing with the outcomes of irrigation and debridement, little is known about which one is the best solution, its composition and its concentration, resulting in a clinical practice based on experience rather than evidence (25). For these reasons, prospective, randomized controlled trials (RCTs) are needed to support the routine use of antiseptics as a stand-alone or adjunct option in the treatment or prevention of PJIs.

In the effort to pursue ever better results in the treatment and prevention of PJIs we devised our own irrigation solution that consists in a combination of dilute HP and PI. The choice of the concentrations of antiseptics (P-I 0.28%; HP 0.58%) in our irrigation solution aims to preserve antimicrobial activity (10) maintaining higher concentrations than those reported by Zubko et al. (6), while lowering the risk of toxicity and embolism (11).

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Synovial and serum levels of NGF in osteoarthritis and rheumatic diseases: a sistematic review

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NGF has raised interest as a target molecule in the treatment of OA, after the clinical evidences that antagonization of NGF axis provides symptoms relief in OA. Thus, we conducted a systematic review of the literature to investigate the evidence of NGF being overexpressed during OA. We conducted a database search on Medline using keywords including NGF, serum, synovial fluid, AND osteoarthritis or arthritis. We included study conducted on human, with serum or synovial specimens and an OA cohort. Nine studies met the inclusion criteria. Serum levels ranged from non-detectable to 153.5±28.6 pg/ml. Synovial fluid levels ranged from non-detectable to nearly 210±82 pg/ml. One study supported the evidence of an increased level of NGF in SF and serum of OA patients. The concentration of NGF reported in these studies is controversial and evidence of overexpression of NGF is low.

Osteoarthritis (OA) is the most common form of arthritis, affecting millions of people worldwide. It is characterized by a gradual loss of articular cartilage in synovial joints that causes pain, reduction of mobility and quality of life. There is growing evidence that articular inflammation, and in particular synovitis, plays a role in the development of the disease. (1) Since then, much effort has been made to describe the molecular patterns characterizing the articular joint environment during OA and a great number of cytokines have been claimed to contribute.

NGF is a member of the neurotrophin family discovered by Rita Levi Montalcini in 1950's (2). It has been demonstrated that it plays a key role in the development, proliferation and differentiation of sympathetic and sensory nerves during the fetal period (3). Mutation with loss of function of NGF or its receptor tyrosin receptor kinase A (TrkA) during this period leads to insensitivity to pain (4).

Moreover, it seems to be of key importance in the early, intermediate and long term generation and maintenance of several type of acute and chronic pain, also during adulthood (5). NGF-TrkA complex modulates the expression of nociceptive receptors, thus enhancing peripheral sensitization. In addition, it also causes post-transcriptional long term changes and stimulates mast cells to express histamine, 5-HT and NGF, forming a positive loop (6). Recently, NGF has raised interest as a target molecule in the treatment of OA, after the clinical evidences that pharmacological antagonization of NGF axis provides symptoms relief in OA and chronic low back pain (CLBP) (7)., Apart from these evidences, though, very little is known about the specific involvement of NGF in OA.

Moreover, these clinical trials have also elicited concerns about major side effects including osteonecrosis (ON) and rapidly progressive OA

Key words: NGF, Osteoarthritis, serum levels, synovial fluid

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(RPOA). In particular, they appear to be dose dependent, leading to a consistent reduction of the dosage in subsequent trials (8). Preclinical studies in experimental models of OA confirmed that the blockade of NGF signaling seem to reduce pain behavior. However, it may promote more rapid cartilage degeneration, synovitis and subchondral bone changes (8).

In addition, there is a consistent number of studies, mostly regarding neurology and psychiatry, that correlate local or systemic reduction of NGF to various condition like schizophrenia (9), (10), peripheral neuropathy (11), dementia (12). These evidences must be considered when considering a systemic drug interfering with NGF axis.

Therefore, postulating the pivotal role of NGF, it is reasonable to assume that levels of NGF should be increased, either locally or systemically, in patients suffering from OA. A better knowledge regarding NGF expression in blood and synovial fluid in course of OA could be helpful in the correct management of patient from diagnosis to therapy. Thus, we conducted a systematic review of the literature to investigate if there is a solid evidence of NGF being overexpressed either locally or systemically during OA.

MATERIALS AND METHODS

We conducted a database search using Medline. The first part of the research was conducted using the following keywords: ((NGF serum) OR (nerve growth factor serum) OR (NGF blood sample) AND ((osteoarthritis) OR (arthritis). A total of 61 abstract were found. The inclusion criteria were cross-sectional studies conducted on human, analysis conducted on serum specimens, at least the presence of an osteoarthritis cohort.

The second part of the study was conducted using the following keywords: (NGF synovia) or (nerve growth factor synovia) OR (NGF synovial fluid) OR (nerve growth factor synovial fluid)) AND ((arthritis) OR (osteoarthritis). A total of 49 abstract were found. Inclusion criteria were cross-sectional studies conducted on human, analysis conducted on synovial fluid (SF) specimens, at least the presence of an osteoarthritis cohort.

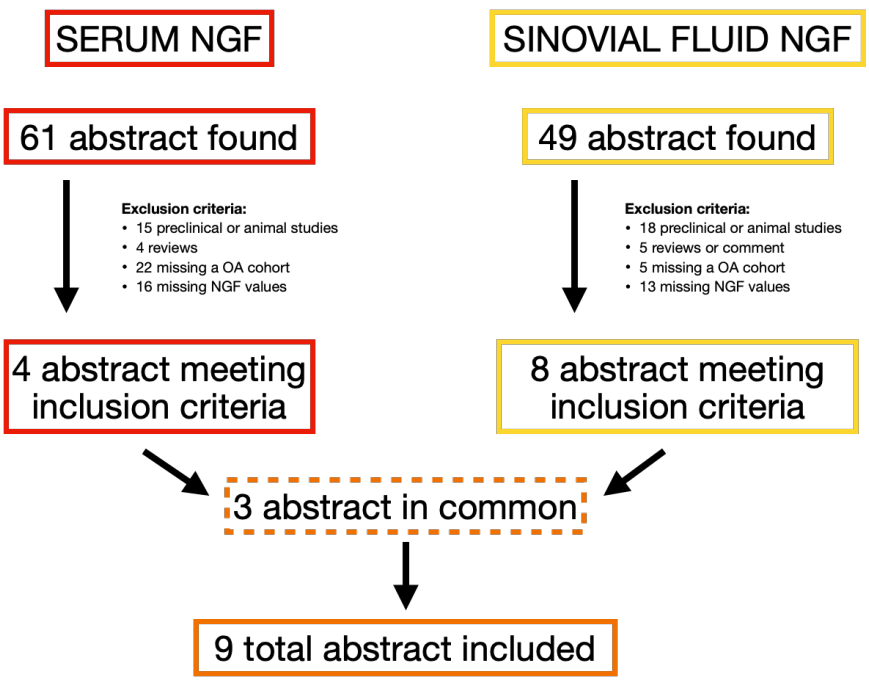

Fig. 1. *Abstracts selection process according to PRISMA protocol.*

At the end of the selection process 9 studies met the inclusion criteria: 4 regarding the first part, 8 for the second, 3 of them being shared among the two groups. Selection process is deeply described in Fig. 1.

RESULTS

NGF serum levels

We found 4 studies meeting the inclusion criteria. Dicou et al (1996) investigated serum and SF levels of NGF and NGF autoantibodies in patients with different types of chronic arthritis such as spondylarthropathy (SpA), rheumatoid arthritis (RA), calcium pyrophosphate dihydrate crystal deposition disease (CPPD) and OA. They obtained sera from 10 patient with SpA, 33 with RA, 11 with CPPD and 27 with OA (Fig 2). They used a two-site ELISA using the monoclonal antimurine NGF antibody 27/21 (Boehringer, Mannheim Biochemica).

In the sera, the estimated NGF values were for the SpA patients 8±16 pg/ml (range 0-40), the RA group 36.9±86 pg/ml (range 0-409), the CPPD group 2.4±7.5 pg/ml (range 0-24) and the OA group 3.7±10 pg/ml (range 0-41). There was no significant difference in the NGF values, though the frequency of detectable NGF was significantly higher in SpA and RA compared to CPPD and OA. Among OA patients only 4 out of 27 had detectable levels of NGF in peripheral blood samples (13).

Rilh et al. (2005) studied the involvement of neurotrophins (NTs) in spondyloarthritis synovitis measuring serum NTs and receptors values in 15 patients with SpA, 15 with RA, 10 with OA and 10 healthy controls. They used a quantitative two-site immunoassay (ELISA) (E&D Systems, Abingdon, UK). They found significative correlation for other NTs but evidenced undetectable levels for NGF in all the four groups (14), Fig. 2.

Montagnoli et al (2017) investigated local and systemic levels of NGF in 20 patients with OA as compared with 15 HC enrolled among patients undergoing routine arthroscopy for minimal meniscal lesions. The two groups were homogeneous for their age (OA: 70.1±5.5; HC: 74.0±3.5). They observed a significantly higher serum NGF level among OA patients (153.5±28.6 pg/ml) as compared with HC (80±11.3 pg/ml). A trend of progressively higher concentration in higher grades of Kellegren - Lawrence Grading Scale (KLS) was also observed. (15)

Chandran et al (2019) aimed to identify soluble

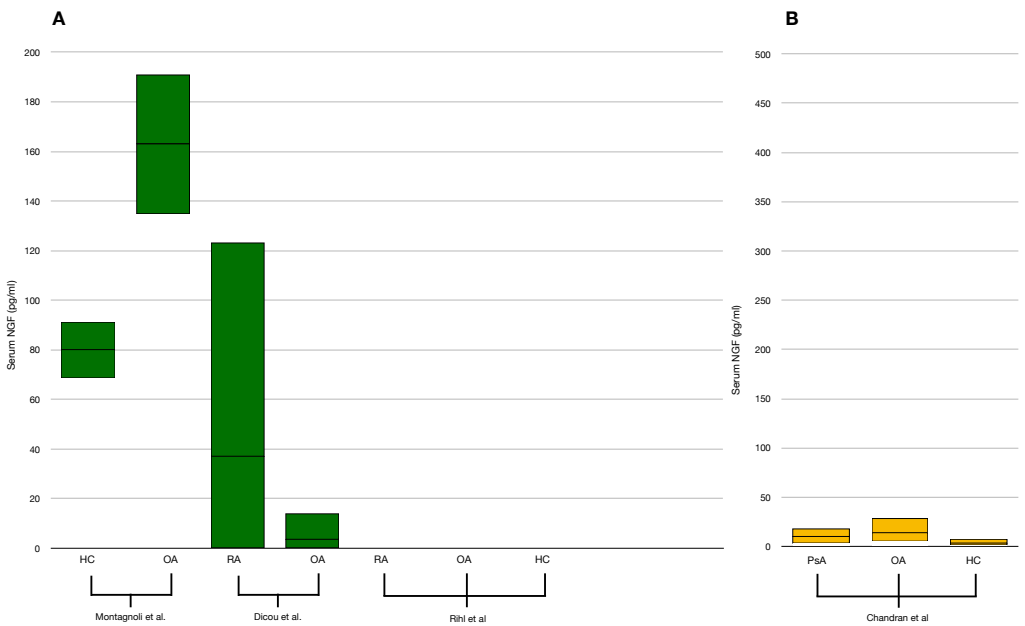

Fig. 2. ***A)****: Serum NGF levels according to different studies. Values are expressed as mean and standard deviation;* ***B)****: Serum NGF levels according to Chandran et al. Values are expressed as median and interquartile interval.*

biomarkers that differentiate PsA from OA. They compared a panel of markers, including NGF, in serum samples from 201 OA patients (57% female; age: median 66, IQ range 58–72), 77 PsA patients (45% female; age: median 43, IQ range 34–54) and 76 healthy controls (HC) (50% female; age: median 35, IQ range 27-44). They found serum NGF levels to be significantly higher in OA patients (median 8.3, IQ range 5.2–15.0) as compared with PsA patients (median 6.1; IQ range 3.8-8.1) and HC (median 2.2; IQ range 1.6-3.7) (16). Results are summarized in Table I.

NGF synovial fluid levels

Some of the studies presented in the first part reported values for SF levels of NGF, as well. Aloe, Montalcini et al (1992) presented the first study regarding the involvement of NGF in chronic arthritis. They enrolled a total of 22 patients with different pathologies: 6 with OA (mean age 66; range 59-76), 8 with RA (mean age 49; range 24-83), 8 with 'other types of chronic arthritis' (4 with PsA, 3 with Ankylosing spondylitis and 1 with juvenile chronic arthritis. Mean age 33; range 14-43) and 2 HC. SF NGF was detectable only in 3 out of 6 samples of patients with OA (range: ND-6 pg/ml), in 7 out of 8 RA samples (range: ND-9 pg/ml) and in all 8 samples from other form of chronic arthritis (range: 2-12 pg/ml). Immunohistochemical studies conducted on synovium tissue samples from OA showed no NGF-positive staining. NGF-positive cells were conversely found in synovium samples from RA (17).

Dicou et al (1996) found values of 121.4±251.8 pg/ml (range 0-785) for the SpA group, 12.5±19.2 pg/ml (range 0-72) for the RA group, 20±54 pg/ml (range 0-163) for the CPPD group and 6.5±19.7 pg/ml (range 0–55) for the OA group. In this case, as well, the frequency of detectable values of NGF was significantly lower in OA as compared with SpA and RA.

Rihl et al. (2005) found detectable levels of NGF only in a minority of SF samples from RA and SpA and none in sample from OA. Barthel et al (2009) measured the mRNA expression of NGF in SF of patients affected by RA (n=9), SpA (n=16) and OA (n=7). Fold of expression for SF NGF were significantly higher in RA (median value: 418) and SpA (median value: 323) as compared with OA (median value: 49) (18), Fig. 3.

Orita et al. (2011) examined the correlation between proinflammatory cytokines in SF and radiographic grading and pain-related scores in 47 consecutive patients with knee OA (26 Female; age 70±2). In particular they found correlation between TNFa and IL-6 and KLS and WOMAC score, but NGF was not detectable in any of the SF samples (19).

Raychaudhuri et al (2011) studied the role of

Table I. *NGF in blood sample according to different studies.*

AUTHOR	METHOD	OA	RA	HC	PsA
Dicou et al. (1996)	ELISA	3.7 ± 10.0 (range 0-41	36.9 ± 86 (range 0-409)	/	/
Rihl et al (2005)	ELISA	ND	ND	ND	/
Montagnoli et al (2017)	ELISA	153.5 ± 28.6	/	80 ± 11.3	/
Chandran et al (2019)	ELISA	8.3 (5.2 – 15.0) *	/	/	6.1 (3.8 – 8.1)*

*Values are expressed in pg/ml. as mean±standard deviation (SD). OA=osteoarthritis; RA=reumathoid arthritis; HC=healthy controls; PsA=Psoriatic arthritis; ND=not detectable. *: values expressed as median and interquartile (IQ) range; /: values are not been examined in the study.*

NGF in inflammatory arthritis and its action on human T lymphocytes and fibroblast-like synovial cells. They observed NGF levels of 395.5±85.2 pg/ml in SF samples from RA (30 patients), 230±35 pg/ml in samples from SpA (20 patients) and 56±6 in OA (30 patients) (20).

Table II. *NGF in synovial fluid according to different studies.*

AUTHOR	METHOD	OA	RA	HC	PsA	SpA
Aloe et al (1992)	ELISA	Range (ND-6)°	Range (ND-9)°	/	/	/
Dicou et al (1996)	ELISA	6.5 ± 19.7	12.5 ± 19.2	/	/	121.4 ± 251.8
Rihl et al (2005)	ELISA	ND	ND	/	/	/
Barthel et al (2008)	Q RT-PCR**	49**	418**	/	/	323**
Orita et al (2011)	ELISA	ND	/	/	/	/
Raychaudhuri et al (2011)	ELISA	56 ± 6	395 ± 85	/	230 ± 35	/
Montagnoli et al (2017)	ELISA	210 ± 82	/	86 ± 7	/	/
Nees et al (2019)	ELISA	9.6 ± 2.9 (range 5.4 – 20.3)	/	/	/	/

*Values are expressed in pg/ml, as mean±standard deviation (SD). OA=osteoarthritis; RA=reumathoid arthritis; HC=healthy controls; PsA=Psoriatic arthritis; ND=not detectable. *: values expressed as median and interquartile (IQ) range; /: values are not been examined in the study; **: values are presented as fold of expression of Mrna.*

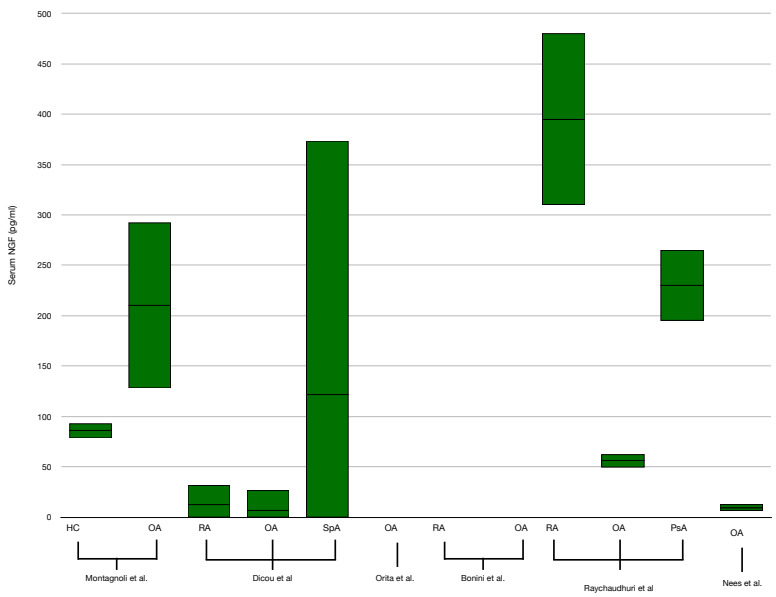

Fig. 3. *Synovial fluid NGF levels according to different studies. Values are expressed as mean and standard deviation. Blank piles represent non detectable concentrations.*

Montagnoli et al. (2017) found SF levels of NGF to be significantly higher in OA (210 ± 82.2 pg/ml) respect to HC (86.2±7.4 pg/ml). Levels were also correlated with the severity of radiological OA graded according to KLS.

Nees et al. (2019) aimed to identify a panel of inflammatory markers of potential clinical relevance in SF sample of patient with OA undergoing either unicompartmental (UC) or bicompartmental (BC) knee arthroplasty. A total of 34 consecutive patients were enrolled (20/34 female, mean age 67±10). SF levels of NGF were found to be 9.6±2.9 pg/ml (range: 5.4-20.3 pg/ml). They were significantly correlated with knee function, as expressed by Oxford Knee Score self-administered questionnaire (OKS-12), but not with pain or OA severity (21). Results are summarized in Table II.

DISCUSSION

The present systematic review aimed to resume the current evidence of the expression of NGF during OA in synovial fluid and serum. To date, NGF is one of the most promising target molecules for the management of pain, especially in patients with OA and CLBP. Despite growing clinical evidence about anti NGF treatments, the burden of NGF in the pathogenesis of OA is yet to be understood.

According to the results of our review, we could notice that preclinical evidence of NGF overexpression in course of OA is controversial. We only found 9 articles meeting our inclusion criteria in the current literature. Moreover, most of these studies are focused on rheumatic diseases such as RA and PsA, with scant consideration to OA.

Resuming, four and eight studies reported respectively the level of serum and synovial NGF in OA patients.

Only one study supported the evidence of an increased level of NGF in SF and serum of OA patients as compared with non-OA controls, even though the statistical sample was limited (15). Regarding serum NGF, all the study reported a wide variability of levels. Montagnoli et al. (15) found blood concentration of NGF from 10 to 40 times higher than Chandran et al. (16) and Dicou et al (13), whereas Rihl et al. (14) found non-detectable values in all the samples. Values ranged from nearly 200 pg/ml to non-detectable.

In SF, Orita et al. (19), Rihl et al. (14) and Aloe et al. (22), reported non-detectable NGF values in nearly all samples from OA patients. Moreover, Dicou et al. (13), Barthel et al. (18) and Raychaudhuri et al. (20). found that the level of synovial NGF in OA was significantly reduced as compared with patients affected by RA, PsA or SpA. The levels measured in the OA populations of all these studies ranged from non-detectable to nearly 300 pg/ml, with apparently contradictory values.

Serrano et al. (1996) (23) and Lang (2003) (24) measured NGF in blood sample of healthy subject. They reported values of 194 pg/ml and 19.68 pg/ml, respectively. Even though values coming from different population are hard to compare, in these studies healthy people reported far higher levels of serum NGF as compared with patients with OA from most of the studies included in our review. However, according to the exclusion criteria of these studies, we cannot exclude that patients in these healthy groups were not affected by OA.

In addition, it is known that NGF is constitutionally expressed in normal chondrocytes and in most of the cartilaginous tissues, (25) and is supposed to have a physiological role. To consider a target therapy against NGF axis, though, it seems reasonable that its eventual increased activity or expression should be pointed out with more solid evidence. A deeper understanding of the NGF role during OA is also required, to indicate whether it has a pathological significance or just a trophic function.

Moreover, considering that adverse effects of anti NGF treatments appear to be dose dependent (8), knowing local and systemic levels of NGF could be useful in the correct management of anti NGF therapy. In conclusion, the concentration of NGF reported in these studies is controversial, and they seem to have very wide and contradictory ranges. Therefore, additional studies with standardized protocols and larger cohort of patients are necessary to establish if OA is associated with defined level of synovial and serum NGF. Moreover, it would be interesting to know if different level of NGF might

be associated with different radiological stages or clinical presentation of OA.

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Mesangiogenic Progenitor Cells and musculoskeletal tissue regeneration: differences between adipose-derived and bone marrow-derived cells?

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Mesangiogenic Progenitor cells (MPCs) have been isolated from human bone marrow mononuclear cells (hBM-MNCs) and attracted particular attention for their ability to efficiently differentiate into exponentially growing mesenchymal stromal cells (MSCs) and toward endothelial lineage, suggesting the term "mesangiogenic". Coupling mesengenesis and angiogenis, MPCs has been hypothesized retaining a great tissue regenerative potential in musculoskeletal tissues regeneration. Bone marrow and adipose tissue (AT) represent most promising adult multipotent cell sources attempting to repair bone and cartilage, with controversial results regarding advantages applying BM- or AT-derived cells. As different culture determinants as well as tissue of origins, could strongly affect regenerative potential of cell preparations, we hypothesize that MPCs counterpart could have a role in defining efficacy of applying a cell-based medicinal product in musculoskeletal tissue repair. Here we present convincing data demonstrating that the *ex vivo* progenitors of MPCs are tissue specific and can be detected exclusively in hBM-MNCs.

After decades from their first identification (1) and characterization (2) in bone marrow (BM), the therapeutic promise of mesenchymal stromal cells (MSCs) is still rising in the regeneration of a broad spectra of injured organs (3). For their differentiation capability, demonstrated *in vitro* and *in vivo*, bone marrow derived MSC have been historically investigated repairing skeletal (4, 5). However, by now the isolation of adult BM-MSCs involves invasive and potentially painful procedures, which also expose to donor site morbidity (6). Consequently to the expanding evidences that MSC-like cells were obtainable from a wide range of adult and perinatal tissues, alternative to bone marrow, a large number of investigations have been performed on the clinical potential of cells derived from more desirable sources (7). Adipose tissue (AT) has some advantages respect to BM, as demonstrated source of MSCs (8) it is abundant and relatively easy to access applying minimal invasive procedures. Unlike BM where MSCs represent a very rare population, AT can provide a consistent number of cells processing few grams of tissue, these cells also showed higher proliferative potential during culture.

However similarly to BM, the isolation of MSCs from AT is highly affected by donor's age, health and site of collection. Moreover, the cell stress induced by the different isolating procedures could strongly contribute to alter the MSC proliferating and differentiating potential (7) In search of more primitive MSC, fetal and perinatal tissues were also investigated, comparing their proliferative and differentiation potential (9).

In 2014, Pacini S. described this heterogeneity of the

Key words: mesangiogenic progenitor cells, bone marrow, musculoskeletal tissues, adipose tissue, cord blood, regenerative medicine

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MSC preparation as the consequence of the combined effects of stochastic fluctuations and deterministic variations, where apparently minimal modification of culture determinants could strongly affect the cell composition and consequently the regenerative potential of a cell-based medicinal product (CBMP) (10). According to this model, in 2009 our group firstly identified Mesangiogenic Progenitor Cells (MPCs) in hBM-MNC cultures simply applying autologous sera as supplement, in place of standard fetal bovine serum (FBS) (11). These MPCs attracted attention for their ability to efficiently differentiate into exponentially growing MSCs, activating the Wnt5/calmodulin signaling pathway (12). From the first observation, MPCs demonstrated also to be able differentiating toward endothelial lineage, suggesting the term "mesangiogenic". Surprisingly, morphology, phenotype and some biological features of MPCs partially resemble the monocytic/macrophagic lineage except for their *in vitro* differentiation capability.

The hypothesis that MPCs could belong to the monocytic/macrophage compartment of human adult bone marrow was corroborated by the identification of the *ex vivo* ancestor of cultured MPCs. Cell sorting experiments demonstrated that the monoblast-like population, described as *Pop#8* and identified by the $CD64^{bright}CD31^{bright}CD14^{neg}CD45^{dim}$ phenotype, represents the unique bone marrrow sub-population able to generate MPCs in culture under selective culture conditions (13). For their peculiar features, coupling mesengenesis and angiogenis, MPCs has been hypothesized retaining a great tissue regenerative potential and clinical value, especially in musculoskeletal tissues regeneration (14, 15).

Here we quantify *Pop#8* population in three different sources: human adult bone marrow (hBM) samples, human stromal vascular fraction (hSVF) and human umbilical cord blood (hUCB).

MATERIALS AND METHODS

Isolation and culture cells from human Bone Marrow (hBM)

The study has been performed according to the declaration of Helsinki and the local ethics committee of "Azienda Ospedaliero-Universitaria Pisana" approved the protocol for human bone marrow (hBM) blood sample collection. After written informed consent, hBM aspirates were obtained from 32 patients (16M/16F, median age: 68, range 52-85) undergoing orthopedic surgery for hip replacement. Briefly, a 20-mL syringe containing 500 I.U. of heparin was used to aspirate 10 mL of fresh bone marrow immediately after femoral neck osteotomy and before femoral reaming. hBM-MNC were isolated as previously described (17). Fresh bone marrow samples were diluted 1:4 in Dulbecco's modified phosphate-buffered saline (D-PBS; Thermo Fisher, Waltham, MA-USA) and gently layered on Ficoll-Paque™ PREMIUM (GE Healthcare, Uppsala, Sweden). Samples were centrifuged at 400g for 25 min and mononuclear cells (hBM-MNCs) were harvested at the interface, filtered on 70 μm filters, and washed twice in D-PBS.

Isolation and culture cells from human Umbilical Cord Blood (hUCB)

The study has been performed according to the declaration of Helsinki and the sample collection protocol was approved by the ethical committee of "Azienda Ospedaliero-Universitaria Pisana". Human UCB was collected from patients undergoing deliveries after receiving informed consent (Santa Chiara Hospital, Pisa, Italy). The human umbilical cord blood (hUCB) samples were harvested from normal term pregnancies (n = 26) between the 37^{th} and the 42^{nd} week of gestation, after both vaginal and caesarean deliveries. The blood was collected simply allowed to flow into heparinized tubes (5000 IU/mL) and processed within 12h. Samples were diluted with Dulbecco's Modified Phosphate Buffer (D-PBS, Thermo Fisher) and hUCB-MNCs collected by density gradient centrifugation using Ficoll-Paque™ PREMIUM (GE Healthcare) as described above.

Isolation and culture cells from human Stromal Vascular Fraction (hSVF)

Seven donors were enrolled in the study. Adipose tissue was collected with prior written consent from patients undergoing cosmetic liposuction. In brief, 250mL of liposuctioned material was extensively washed with equal volumes of phosphate buffered saline (PBS) to remove erythrocytes and then centrifuged for 5 min at 600g to separate the fat from the oil and liquid phases. After washing, fat was combined vol/vol with 125 CDU/ml type IV Collagenase (Thermo Fisher) and incubated

for 1 hour at 37°C in a shaking bath water. Samples were filtered through a 100 µm and the human stromal vascular fraction (hSVF) was harvested by centrifugation at 600g for 10 min. The resulting pellet was then resuspended and hSVF-MNCs were isolated by gradient centrifugation.

FlowCytometry

150'000 BM-MNCs, hCB-MNC, hSVF were incubated with anti-human CD64 FITC-conjugated, CD31 PE/Cy7-conjugated, CD14 VioGreen®-conjugated and anti-human CD45 VioBlue®-conjugated (MiltenyiBiotec, BergischGladbach,Germany) for 30′ at 4°C in the dark, and washed twice in MACS Quant® Running Buffer (MiltenyiBiotec). Data were acquired using MACS Quant® flow cytometer and analyzed by MACS Quantify® Analysis Software (MiltenyiBiotec).

Flow cytometry of freshly detached cells from primary cultures were also performed as described above applaying anti-human CD90 FITC-conjugated, CD73 PE-conjugated, CD31 PE/Cy7-conjugated, CD14 VioGreen®-conjugated and anti-human CD45 VioBlue®-conjugated (MiltenyiBiotec).

RESULTS

Frequency of Pop#8

Multicolor flow cytometry of freshly isolated hMNCs from different sources, was applied to quantify the *Pop#8* subpopulation. According to previously described *Pop#8* immunophenotype (13), *ex vivo* MPC progenitors were identified within $CD64^{bright}CD31^{bright}$ population detected in both hBM-MNCs and hUCB-MNCs, not in hSVF-MNCs where a double-positive population were detected but expressing low levels of CD31. However, the genuine *Pop#8* population defined as $CD64^{bright}$ $CD31^{bright}CD14^{neg}CD45^{dim}$ (green in Fig. 1A) was detectable only in hBM-MNCs in a mean percentage similar to what previously reported (1.60 ± 0.12%, Fig. 1B).

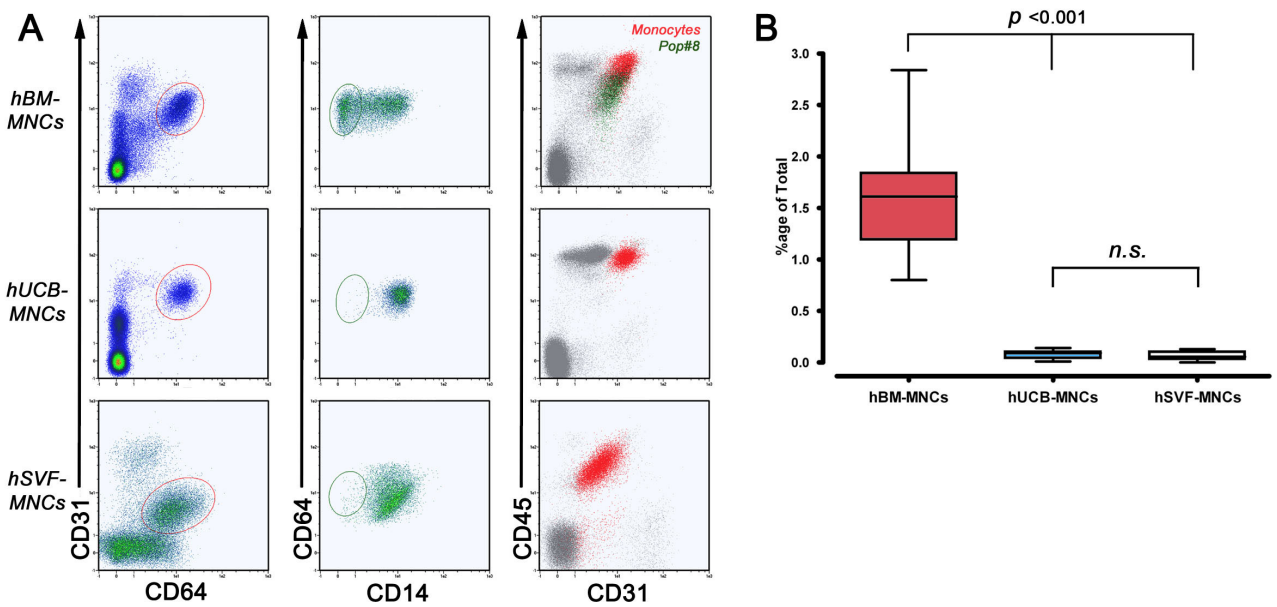

Fig. 1. *Flow cytometry quantification of MPC ex vivo progenitors (Pop#8);* ***A****): Scattergrams from hBM- and hUCB-derived MNCs resulted very similar both reporting evident* $CD64^{bright}CD31^{bright}$ *population (red gates). Most of these cells showed phenotype of mature monocyte for the consistent expression of CD14. However, the* $CD64^{bright}CD31^{bright}CD45^{dim}CD14^{neg}$ *(knowed as Pop#8, green gates) were detectable only in hBM-MNCs. hSVF-MNCs showed mildly different expression levels of the investigated markers, possibly due to the different isolating procedure (rappresentative samples are showed);* ***B****): Mean percentage of Pop#8 in hBM-MNCs resulted around 1.5% of the total.*

In hUCB-MNCs, almost the whole $CD64^{bright}CD31^{bright}$ population was represented by CD14-positive mature monocytes (red in Fig. 1A) as well as the double-positive population detected in the hSVF-MNCs expressed CD14 and CD45 even if at a lower intensity. Differences in the level of antigens expression reported in hSVF-MNCs could be ascribed to the different isolating procedure compared to hBM- and hUCB-MNCs.

DISCUSSION

MSCs and their *ex vivo* ancestors hold great promise for the treatment of bone and cartilage defect (17) To date, BM-derived mononuclear cells and stromal vascular fraction from adipose tissue are still the main source of adult multipotent cells to apply especially in autologous cell-based therapies (18) Both BM-MSCs and AT-MSCs have been used for the successful repair of various bone defects, including critical size defects in the long bone (19), hard palate reconstruction (20), and craniomaxillofacial defects (21).

Many studies have shown that BM-MSCs and AT-MSCs share similar features, including morphology and cell surface markers, but significant biologic differences have been found concerning their proliferation and differentiation capacities. However, controversies have been reported, where some studies indicate that the potential of AT-derived cells are more effective than or as effective as that of BM-derived, while other studies conclude that BM is superior to AT (22, 23).

Even if an increased efficacy in cell isolation, expansion and characterization lead to a consistent number of studies applying adult multipotent cells in various disease models, most of the clinical and pre-clinical investigation showed disappointing outcomes, related to the efficacy endpoints to be reached with the lack of a long-lasting and consolidated repair of the defects (24).

Taking into account these considerations, MPCs have attracted attention for the regeneration of musculoskeletal tissues. Together with an affordable method isolating these cells, the retention of both mesengenic and vasculogenic potential make MPCs a very promising tool in repairing large bone and cartilage defects. However, as a definitive demonstration of MPC clinical value in animal models is still lacking, MPCs and their *in vivo* progenitors (Pop#8) have been not investigated in the clinical application of application of adult multipotent cells.

Conversely, MSCs have been used in bone tissue engineering to improve the therapeutic options for enhancing bone repair, as these cells have been studied in a wide variety of animal species. Analyzing the clinical readiness of MSCs and their progenitors, their application in the treatment of orthopaedic diseases and defects is dominant and BM-derived cells still represent the most applied cells engineering a cell-based medicinal product (24). This is probably due to several pre-clinical studies demonstrating that bone marrow-derived cells are more effective in the regeneration and repair of skeletal tissues respect to alternative sources (25). As showed by Authors, AT-MSCs demonstrated *in vivo* an inferior osteogenesis capacity and a superior angiogenesis capacity compared with human bone marrow stem cells (BMSC) (25).

These findings have important implications for the use of AT-MSCs in bone repair because the advantages of their use may be negated by their lack of prerequisite for osteogenic differentiation prior to transplantation. In the present studies we demonstrated that AT-MSCs do not possess any intrinsic vasculogenic potential corroborating the idea that AT-MSCs could support local angiogenesis by the reported secretion of specific factor. Nonetheless, this could represent a further limiting factor in regenerating naturally low vascularized tissues as bone and cartilage.

Concluding, our results demonstrated that the monocytic/macrophagic counterparts of hSVF-derived mononuclear cells are exclusively constituted by $CD64^{bright}$ $CD31^{bright}CD14^{+}CD45^{bright}$ normal tissue resident monocytes and macrophages, which were co-isolated with MSC-like cells under MPC-selective culture without providing any vasculogenic properties to the cell preparation. Conversely, as reported previously the $CD64^{bright}$ $CD31^{bright}CD14^{neg}CD45^{dim}$ MPC progenitors, known

as *Pop#8* population, were consistently detected in hBM-MNCs allowing the isolation of MPC under selective culture conditions (13).

Here we hypothesized that the suspected superior performance of both hBM concentrates and hBM-MSCs, respect to adipose-derived cells in skeletal tissue regeneration, could be explained by the presence of MPCs and/or their progenitors that could trigger new blood vessel formation in the early phases coupled with the essential chondrogenic and osteogenic differentiation capability.

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Thumb basal joint interpositional arthroplasty: a systematic review

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The aim of this systematic review was to analyze the failure rates among different trapeziometacarpal interposition implants used to treat thumb basal joint osteoarthritis. We searched Medline (PubMed), Web of Science and Scopus databases, to identify articles reporting on thumb interpositional arthroplasty, in English literature. We excluded studies with less than 35 cases and with a follow-up shorter than 24 months. Twenty-one studies were included. We assessed the quality of the studies using the Coleman Methodological Score. The mean quality of the studies was moderate. The total number of procedures included in this review was 1205. The failure rate for interposition implants was 11%. The main long-term complication was dislocation, which is also the major reason for revision.

Trapeziometacarpal (TMC) joint arthritis can cause severe pain, weakness, and deformity resulting in marked disability. Conservative treatment is involved in the early stages, but, as the condition progresses with persistence of symptoms, surgical intervention is indicated. A variety of procedures have been described, including metacarpal osteotomy, carpometacarpal (CMC) arthrodesis, joint replacement, and trapeziectomy with or without tendon interposition (TI), ligament reconstruction (LR), or ligament reconstruction combined with tendon interposition (LRTI). To date, no single method has emerged as superior, although each method has specific advantages and disadvantages (1). Among them, TMC replacement has been attractive, because the preservation of trapezoidal space height could result in greater thumb strength (2). Currently, a variety of TMC implants are available, varying in design concept as well as materials used. Nevertheless, these types of implants have reported high rates of complications, revisions, and failures. The aim of this systematic review is to analyze the failure rates among TMC interposition implants used to treat CMC osteoarthritis.

MATERIALS AND METHODS

We performed a systematic review of the literature according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines with a PRISMA checklist and algorithm (3). A literature search was performed combining the following keywords: 'thumb basal joint', 'trapeziometacarpal joint', 'first carpometacarpal joint', 'osteoarthritis (OA)', 'implant replacement', 'interposition implant' and 'interpositional arthoplasty'.

Medline (PubMed), Web of Science and Scopus were accessed up to 3rd of May 2020. We included articles in English only. Biomechanical studies, studies on animals or cadavers, technical notes, letters to the editor, review articles and instructional courses were excluded. Moreover, we excluded studies with less than 35 cases

Key words: rizoarthrosis; thumb basal joint; interposition; arthroplasty; replacement

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and with a follow-up shorter than 24 months. Two authors independently assessed the abstract of each publication. When it was not possible to include or exclude an article based on the abstract, a full-text version of the article was downloaded. If the abstract was not available, the article was excluded from the study. In addition, we retrieved the reference list of each selected article to identify additional studies missed at the first electronic search.

The two investigators assessed each study according to the Coleman Methodological Score (CMS) (4) ranging from 0 to 100, whereas a 100 score is referred to as the best study design. Both investigators performed the CMS assessment twice, with a 10-day interval between the two evaluations. They then discussed the scores where more than a two-point difference was present, until a consensus was reached. Data on demographic features, operative readings, follow-up periods, type and numbers of complications and survival rate of the procedure were recorded.

RESULTS

A total of 543 studies were identified at the first research and 80 studies were selected based on the abstract. Finally, 21 articles were included in our systematic review (Fig. 1).

The total number of procedures was 1205 with a mean follow-up of 65.5 months (Table I). The mean age at time of surgery was 58.5 years, in three studies was not reported (16, 18, 22). We utilized the CMS to evaluate the quality of the studies: a CMS >85 is considered excellent, 70-84 good, 50-69 moderate and <50 poor. The mean CMS was 60 (range 42-77) indicating a moderate methodological quality (Table I).

All studies were case series. Failure rate was 7%, for a total of 84 cases of revision. One study (21) did not state the number of revisions.

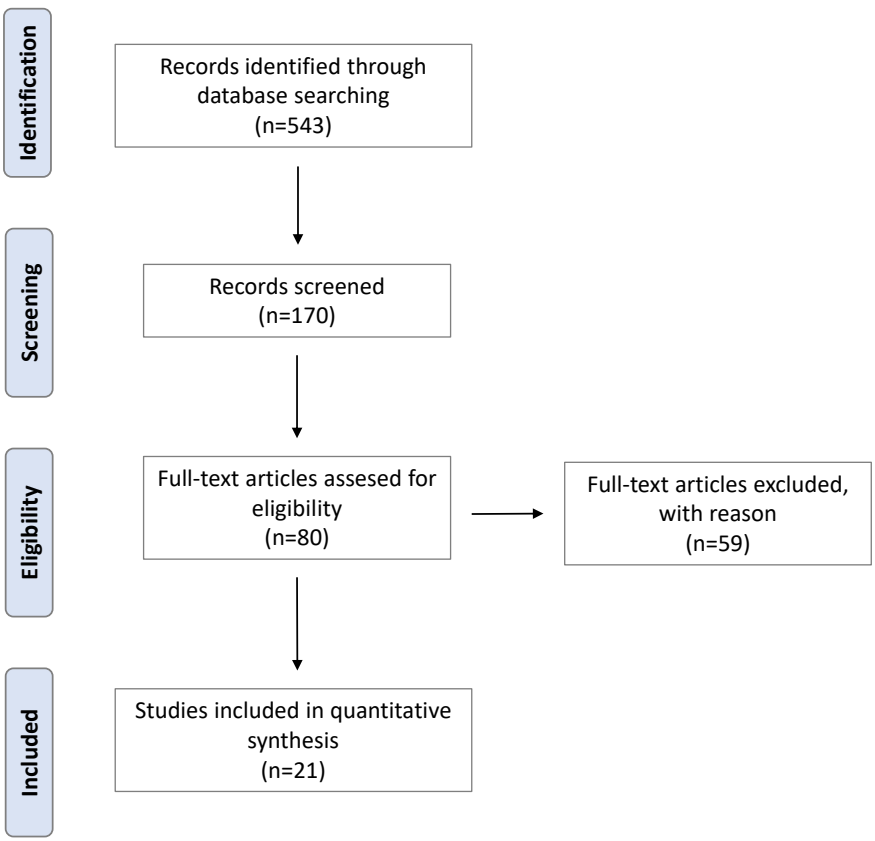

Fig. 1. *PRISMA flowchart. A total of 543 studies were identified at the first research and 170 studies were selected based on the abstract. Finally, 21 articles were included in our systematic review.*

One article (12) had no cases of failure. The most common complication was dislocation (11%). Data are resumed in table I.

DISCUSSION

A variety of surgical techniques have been described for the treatment of TMC osteoarthritis, but none have been proven to be superior to the other (1). Prosthetic arthroplasty has the theoretical advantage to maintain thumb length and prevent proximal migration of the first metacarpal, with restoration of functional range of motion and strength, and pain relief (1). Although, superior short-term outcomes, such as increased grip and pinch and faster recovery after implant arthroplasty, compared with non-

Table I. *Characteristics, failure rates and complications reported by the studies on interposition implant included in this systematic review.*

Study (type of implant)	Number of procedures (patients)	Mean age (years)	Mean follow-up (months)	Failure rate	Most common complication	Coleman Methodological Score (CMS)
Russo et al, 2016 (Pyrocardan implant) (5)	36(36)	58.5	31.5	5% (2/32)	Dislocation	68
Odella et al, 2014 (Pyrocardan Implant, PyroDisk) (6)	59(59)	62	42	7% (4/59)	Persisting pain	55
Smeraglia et al, 2020 (Pyrodisk) (7)	46(46)	62	113	6% (3/46)	Dislocation	77
Bell et al, 2011 (Artelon Spacer) (8)	49(46)	57.8	48	8% (4/49)	Persisting pain	53
Adams et al, 2009 (Orthosphere) (9)	50(49)	59	36	6% (3/50)	Persisting pain	58
Ashworth et al, 1977 (Ashworth implant) (10)	49(42)	55	31	4% (2/49)	Fracture implant	47
Kessler et al, 1984 (Proplast stabilized trapezial Implant) (11)	45(40)	40	24.7	9% (4/45)	Dislocation	50

Agout et al, 2016 (Pi2) (12)	42(39)	63	125.5	0	Osteolysis	77
Szalay et al, 2013 (Pi2) (13)	60(60)	58.5	23.6	10% (6/60)	Dislocation	61
Van Aaken et al, 2016 (Pi2) (14)	45(41)	60	29	26% (12/45)	Dislocation	51
Jewell et al, 2011 (Swanson trapezium implant) (15)	86(63)	66	46	1% (1/86)	Dislocation	66
Bezwada et al, 2002 (Swanson trapezium implant) (16)	62(58)	NS	196	6% (4/62)	Fracture implant	70
van Cappelle et al, 2001 (Swanson trapezium implant) (17)	45(35)	61	165.6	26% (12/45)	Dislocation	69
Lovell et al, 1999 (Swanson trapezium implant) (18)	58(NS)	NS	62	7% (4/58)	Subluxation	52
Freeman and Homer, 1992 (Swanson trapezium implant) (19)	43(37)	60.5	66	9% (4/43)	Foreign body reaction	65
Creighton et al, 1991 (Swanson trapezium implant) (20)	151(124)	62	51	1% (2/151)	Osteolysis	57
Sollerman et al, 1988 (Swanson trapezium implant) (21)	39(33)	58	144	NS	Subluxation	61
Hay et al, 1988 (Swanson trapezium implant) (22)	64(52)	58	52.8	8% (5/64)	Subluxation	42
Lister et al, 1977 (Swanson trapezium implant) (23)	36(31)	NS	27.4	16% (6/36)	Subluxation	44
Helal and McPherson, 1989 (Helal prosthesis) (24)	40(31)	55.4	32	10% (4/40)	Persisting pain	58
Kokkalis et al, 2009 (Acellular dermal allograft (GraftJacket) (25)	100(89)	57	30	2% (2/100)	Persistin pain	73

***NS**= not stated*

implant techniques (trapeziectomy, trapeziectomy with ligament reconstruction and tendon interposition, joint fusion) have been reported (1), it is evident that many of the implants carry higher complication and failure rates (Table I).

Currently, many different implants are available for the treatment of base thumb OA. This systematic review focuses on failure rates of TMC interposition implants reported in the English literature. Although most of the studies were retrospective case series, we wanted to increase the quality of data excluding studies with small numbers, and less than 2 years follow-up. In the 1960s, the trapezial Silastic implant (Swanson) was developed. This replacement arthroplasty filled the space produced by trapezial excision, but it was associated with high long-term complications such as subluxation, fractures, and silicone synovitis (15-23). In recent years pyrolytic carbon has been used as a new material for TMC implants. It is a synthetic material with a module of elasticity like that of cortical bone, resulting as suitable for interposition arthroplasty.

Pyrocarbon interposition implants theoretically avoid the problems of polyethylene wear and metallosis seen with the older generation of cemented implants (7). Different implants made from this material are available for interposition arthroplasty. Pyrocardan implant (BioProfile/Tornier) and Pi2 prosthesis (BioProfile/Tornier), obtained significant improvement in pain and subjective scores (5, 12), but with high complication rates even in the short term (13, 14). Odella et al. (6) compared to an anatomically shaped pyrolytic implant (Pyrocardan) and a non-anatomically shaped one (PyroDisk). They observed good outcomes with both implants but a higher rate of complications occured, when using Pyrocardan. They think it could be since Pyrocardan implantation is more difficult because it demands greater precision in bone resection (6).

Smeraglia et al. (7) reported a survival rate of the PyroDisk implant of 94% in 8 years, making the implant attractive especially in younger patients (aged below 60 years), in fact it could be used as a time-buying procedure because it does not 'burn any bridges' (7). Although Agout et al. (12) reported no cases of failure, Van Aaken et al. (14) reported an unacceptable revision rate of the Pi2 implant of 27% (12/45), with a median time of implant failure at 10 months. This is consistent with the previous studies reporting high revision rates (13). Additionally, the implant subluxation rate was 62% (13 of 21 unremoved implants). However, they observed no significant difference in key pinch between patients with subluxated implants and patients with in-place implants, so they concluded that subluxation and the consequent reduction in scaphometacarpal distance, have limited impact on strength (14).

Nevertheless, the use of prosthetic implant in TMC arthritis remains controversial and it should be indicated in selected cases. The implants, independently from the design or the material, do not show significant clinical improvements to justify the cost of the implant itself.

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Trabecular titanium tailored implants in complex acetabular revision surgeries: our experience at minimum 3 years follow-up

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Hip revision surgery in cases with previous multiple reconstructive procedures is a challenging procedure. A custom-made product could be useful to obtain good results. Four cases (2 men and 2 women) of acetabular customized Trabecular Titanium (TT) implants made by 3D printed technology were performed. The mean age at surgery was 51.5 years (range 25-72). Patients were reviewed clinically and radiographically at follow-up. Mean Harris Hip Score increased significantly from 13.9 (range 6.9-20.6) preoperatively to 75.8 (range 53.9-94) at last follow-up (mean 43.5, range: 36-59), showing an improvement in terms of both pain relief, function and joint mobility. Radiographically neither signs of instability, migration nor tilting were observed. TT custom-implant made by high performance of the 3D-printing technology, system modularity, and patient-specific surgical tools permitted good clinical results and an effective restoration of the biomechanical joint parameters in these complex revision cases.

Failure of total hip arthroplasty is often associated with significant acetabular bone stock loss. Norwegian Arthroplasty Register reported that the failure rate of revision hip arthroplasty is 25.6% versus 11.4% for primary THA at 10 years follow-up, especially for acetabular component revision (1). Yu et al. found that aseptic loosening was the first cause of implant failure and that the indications for both primary and re-revision surgery were acetabular loosening in 20% and 24% of cases respectively (2).

Several classification systems have been developed to define bone defects of the acetabulum. There is no consensus as to which system of classification to use, and the utilization is geographically dependent. The American Academy of Orthopaedic Surgeons (AAOS) recommends the classification of D'Antonio (3), whereas European surgeons tend to adopt the system developed by Paprosky (4).

A made production system in Electron-Beam Melting (EBM) technology based on an analysis of patients' preoperative CT scans could be used to create custom implants useful in hip revision surgery in presence of severe acetabular bone defects.

MATERIALS AND METHODS

Four cases (2 men and 2 women) of acetabular customized implants were performed in a single center by a single senior surgeon. The mean age at surgery was 51.5 years (range 25-72). The bone loss was classified according to the Paprosky classification as IIIA in 2 cases and IIIB in the other 2 cases. All patients must be assessed clinically and with radiographic images before surgery. This included standard X-rays of the hip, AP pelvis, as well as CT scans which are mandatory to create bone custom models.

The CT scan protocol consisted of images that start from the top of iliac crest up to mid-femur, or at least 3 cm below the existing femoral implant. The optimal

Keywords: custom made, tailored implants, 3D printing, severe acetabular bone defects

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data involves images with a thickness of 1.0 mm and a spacing of 0.8 mm (maximum acceptable are 2.0 mm of thickness and 2.0 mm of spacing), with metal subtraction software with the uncompressed data recorded to send to the implant manufacturer. The CT scan slide data are used to create a computerized, three-dimensional (3D) reconstruction of the patient's hemipelvis. The implants are developed by Lima Corporate Spa using 3D printing additive manufacturing technology (EBM): a high-energy focused beam is used to locally melt metallic powders layer upon layer in a one-step manufacturing process.

The structural continuity between the external highly porous trabecular surface and the inner bulk is obtained. The 3D Printing (EBM) technology permits creating an extremely flexible patient matching implant and instrument, with material performances not viable with the standard manufacturing process. Dedicated visual 3D tools and instrumentations were developed to improve implants' congruency according to the preoperative plan. To enhanced primary stability and tailored on patient's anatomy through press-fit, high friction performances of the Trabecular Titanium matrix were used. The use of bone screws and their position was designed to improve the construct stability, even in critical bone conditions, avoiding implant stress shielding and allowing bone integration.

The cup orientation is established by setting the abduction and anteversion angles of the cup, and it is based on patient-specific considerations, including leg length discrepancy, planned retention or revision of the femoral component, length of contralateral leg, and cup size. The abduction angle generally is targeted at 40° to 45° and is established using the plane of the obturator foramen as a reference. The anteversion angle is recognized using the plane of the iliac wing and the obturator foramen as references.

The posterior hip approach was used in all cases for correct exposure and visualization of the pelvis. It is possible to identify and trace the sciatic nerve from the greater sciatic notch to the ischium. After dislocation of the hip, in one case the femoral stem was removed from the top of the femur. In the other 3 cases, the femoral component is retained, and the gluteus minimum and gluteus medius are elevated from the iliac bone and space created between the muscle and the ilium, and the taper of the stem could be placed into this space. The previous cup must be removed, without sacrificing further bone. If it was necessary for the visualization of the ilium, the gluteus medius and minimus was elevated off the wing of the ilium, to protect the superior gluteal artery and nerve.

A sterilized 3D pelvic model and a cup model could be used as guiding reference intraoperatively. Acetabular pseudomembranes were removed and a necrotic bone reamed according to the 3D model and the preoperative planning. An allograft to fill a bone defect was used only in 1 case, to fill some remaining imperfections of contact with the construct, to achieve the best possible contact between the new cup and the supporting bone. The 3D trial models of the cup are available, and it is useful to check the bone-implant contact area and the correct position of the screw's holes and paths. Cleaning of the bony bed for the new implant from remaining cement, screws, metal, is mandatory to achieve the same clean surface as planned by the CT reconstruction.

After the correct positioning of the custom cup, it must be fixed with screws that have a diameter, direction, and length already defined. The screws (insertion points, paths, screwing direction) was planned and discussed with the engineers during the planning because not all insertion points can be intraoperatively reached. The custom cups used allowed several internal modularity options ('face changers', or spacers) which included standard or protruded liners (+5 mm offset), with straight or angled (10°-20°) spacers. In our series, face changers were used in 3 cases to restore correct offset and a dual mobility liner was employed in 1 case.

The post-operative rehabilitation included immediate mobilization with protected weight-bearing using crutches for the first 30 to 60 days. Due to the absence or to the small quantity of bone grafting the weight-bearing is not contraindicated.

Radiological evaluation including AP pelvis X-rays and axial view of the affected hip was performed postoperatively, at 6 weeks, 6 months, and 1 year, and, thereafter, at 1-year intervals. A post-operative CT-scan was performed in 2 cases to evaluate the congruence between the bone and the implant and the direction of the screws. Clinical evaluation could be performed by the Harris Hip Score (HHS).

RESULTS

No signs of miss-match between intraoperative bone conditions and pre-operative planning were observed. Biomechanical parameters were

restored also by using internal modularity (Fig. 1). Incompatibility or impingement between the stems and new acetabular component was not observed and stem revision was performed in one case.

On-table stability proved excellent and no intraoperative complications were observed. Mean Harris Hip Score increased significantly from 13.9 (range 6.9-20.6) preoperatively to 75.8 (range 53.9-94) at the last follow-up (mean 43.5, range: 36-59), showing an improvement in terms of both pain relief, function and joint mobility. Radiographically neither signs of instability, migration nor tilting were observed (Fig. 2). No case of dislocation nor infection were recorded.

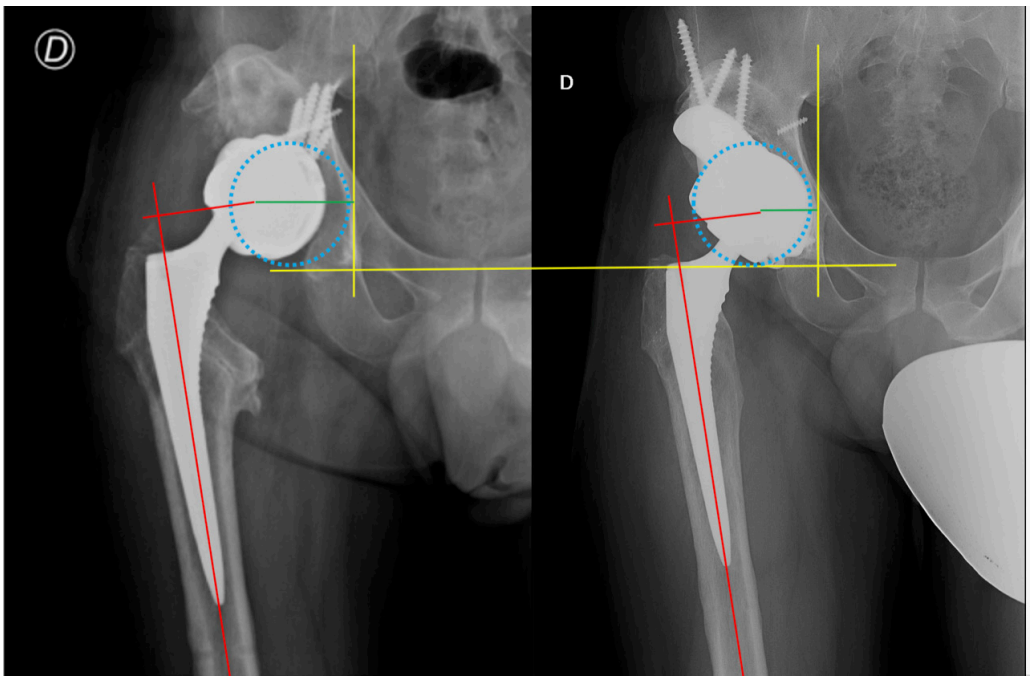
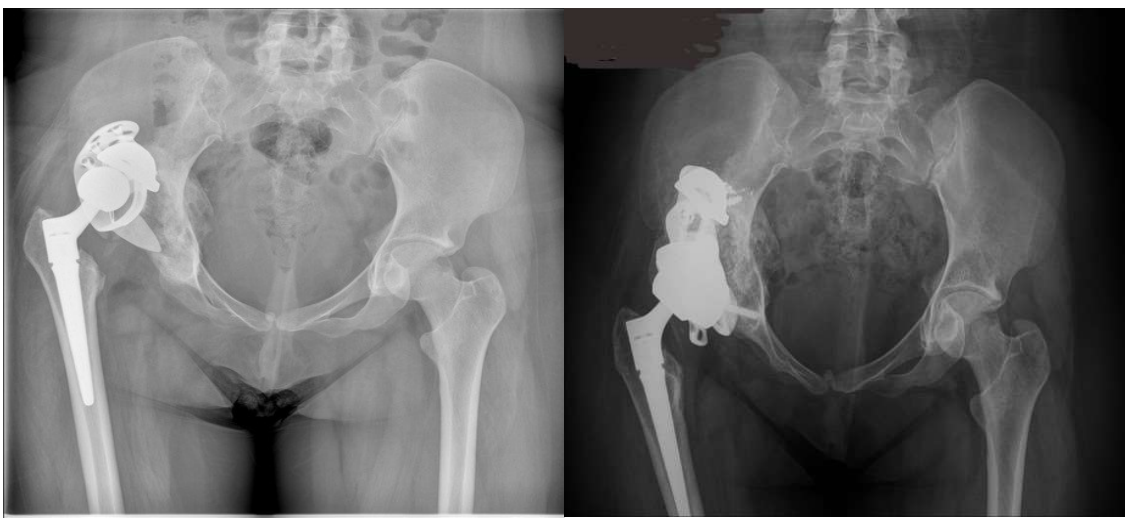


Fig. 1. *Pre-operative and 39 months follow-up x-rays of custom implant: biomechanical parameters are restored.*



a. b.

Fig. 2. *C.M, 25 y, W;* ***a****): Outcome of acetabular necrosis with 2 previous revision surgery;* ***b****): Aseptic loosening of Burch Schneider cage and 40 months follow up after revision using Trabecular Titanium custom made cup.*

DISCUSSION

Total hip revision surgery in cases of previous multiple reconstructive procedures is a challenging procedure due to difficulties in the treatment of huge bone defects with standard revision of prosthetic combinations.

In a recent review, Chiarlone et al. describe acetabular aseptic loosening (AL) like the main indication for revision of total hip arthroplasty with a custom implant, followed by implant failure, osteolysis, PJI, multiple dislocations, instability, metallosis, dysplasia, Girdlestone, tumor and acetabular fractures (5). Christie et al. (6) reported no triflange cup removed and Harris hip scores improved from a preoperative mean of 33.3 points to a postoperative mean of 82.1 points in a series of 67 hips in 65 patients with an average follow-up of 53 months (range, 24-107 months).

Recently, the literature on the failures of the custom-made implants became available. The most common causes of revision are dislocation, aseptic loosening, infection, and mechanical failure. De Martino et al. (7) reported 11% of dislocation, 6% of deep infection, and 1.7% of aseptic loosening in a review enrolling 579 cases. In a single series of 95 complex acetabular reconstruction with custom triflange cup, Berend et al (8) described that the implant was removed in seven hips (7.37%), and a further triflange implant was used in four and an excision arthroplasty was performed in three after the failure of reimplantation due to infection.

Mechanical failure of a custom system is also possible, and it could occur due to screws breakage, flange breakage, cement detachment, or cup breakage. A small number of data are available in the literature on the possibility to revise custom implants. In our opinion it is very important to prevent the possibility of custom implant failure using porous materials, good manufacturing system/technology, modularity, an adequate level of constraint associated to abductor repair/reconstruction if needed, and maniacal planning (to identify cup position, screws direction, good bone available if it is possible); the surgeons must be skilled and the surgery must not last too long, to reduce the risk of infection.

In our small series, a detailed anatomical reconstruction, in-depth preoperative planning, custom-implant design, high performance of the 3D-printing technology, system modularity, and patient-specific surgical tools permitted a good clinical and radiographic results with an effective restoration of the biomechanical joint parameters in these complex revision cases. The optimal primary stability of the implants promoted early osseointegration with the remaining bone stock. Further studies shall be necessary to assess the performance of these Implants at the long-term follow-up.

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Gut microbiota and osteoarthritis: a deep insight into a new vision of the disease

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Osteoarthritis (OA) is a multifactorial disease, whose exact pathogenesis is still unclear. In recent years, the gut microbiota (GM) has shown to modulate not only local processes but also systemic responses. This narrative review aims to summarize the recent evidence about the link between gut dysbiosis and OA onset and define a potential preventive and therapeutic strategy. OA symptomatic expression, resulting from the complex interplay between mechanical and biological factors, might be enhanced by systemic low-grade inflammation. It is reported several OA-related risk factors are linked to a systemic inflammatory status and potential GM dysfunctions. Moreover, recent studies have demonstrated the presence of lipopolysaccharides, proteoglycan and bacterial nucleic acids in the synovial fluid of patients undergoing total knee arthroplasty. In the future, microbiota profiling could help predict OA progression and, at the same time, GM could be a potential target in the treatment and prevention of OA.

Osteoarthritis (OA) is the most prevalent degenerative joint disease and a leading cause of pain and disability in middle-aged and elderly people (1). Characterized by cartilage degeneration, intra-articular synovial inflammation and subchondral osteosclerosis, OA causes joint pain, swelling, stiffness, deformity and loss of function, thus progressively affecting the working ability and the social life of the patient (2)incidence and mortality risk. The prevalence and incidence of symptomatic, radiographic and self-reported hip or knee OA were included. Three levels of severity were defined to derive disability weights (DWs.

Unfortunately, the onset of the OA symptoms usually occurs after the irreversible joint changes have developed (3). Therefore, in a significant number of patients, arthroplasty still is the most successful procedure, with a reported high patient's satisfaction rate (4).

OA is a multifactorial disease, hence several genetic and environmental risk factors promote its development, including age, gender, ethnicity, body mass index (BMI), physical activity and muscle weakness but the exact pathogenesis of is still unclear (5).

Therefore, in the Era of Medicine 4.0, several research groups are using omics approaches to better understand the mechanisms involved in the OA pathogenesis and progression (6). From a translational point of view, this line of research could provide useful data to prevent OA progression

Keywords: osteoarthritis; synovial fluid; omics; microbiota; dysbiosis; inflammation; Medicine 4.0

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(enhancement medicine) or selectively treat early OA (precision medicine), thus hopefully reducing the number of patients' candidate to arthroplasty.

In this context, the study of gut microbiota is gaining increasing importance. There is growing evidence that gut dysbiosis, contributing to the genesis of a chronic low-grade systemic inflammation, might promote together with the onset of metabolic syndrome, also musculoskeletal diseases (5, 7). Consequently, the concept of metabolic osteoarthritis, i.e. a metabolic syndrome-related OA, has been recently introduced (8). This hypothesis could explain, along with the increased general population longevity, the high OA prevalence observed in developed countries (9-11). This narrative review aims to summarize (1) the recent evidence about the link between gut dysbiosis and OA onset and (2) define a potential preventive and therapeutic strategy.

Gut microbiota and body homeostasis

The gastrointestinal tract has a huge commensal population of microorganisms since it is colonized by more than 100 trillion microbes, who have symbiotically evolved to habituate the human gut (12). Although the microbiota composition varies in each person, four main classes constitute the physiologic microbiota, i.e. *Firmicutes, Bacteroides, Proteobacteria,* and *Actinobacteria* (12).

The Gut Microbiota (GM) plays a major role in maintaining body homeostasis, since it is involved in several physiological processes, including digestion and absorption, metabolites secretion and mucosal barrier function protection (13). However, the physiologic gut microbiota could be affected by prolonged antibiotic assumption, gastrointestinal diseases and wrong dietary habits, thus promoting a condition of gut dysbiosis (12, 14).

It is reported dysbiosis could affect life expectancy and long-term quality of life, especially in elderly people (12). In recent years, the gut microbiota has shown to modulate not only local processes but also systemic responses, thus its role has been investigated in several multifactorial diseases including metabolic syndrome, diabetes mellitus type 1, cancer, multiple sclerosis, rheumatoid arthritis, spondylarthritis, autoimmune hepatitis, osteoporosis and osteoarthritis (9).

Gut dysbiosis and OA-related risk factors

OA symptomatic expression, resulting from the complex interplay between mechanical and biological factors, might be enhanced by systemic low-grade inflammation (6). It is reported several OA-related risk factors - including ageing, obesity and lifestyle - are linked to a systemic inflammatory status and potential gut microbiota (GM) dysfunctions (15).

Ageing progression causes relevant GM composition changes, including reduced bacterial diversity and variations in dominant GM species, that have been associated with chronic systemic inflammation (16). Therefore, the reduction of GM species with anti-inflammatory properties (i.e. Clostridium cluster XIVa species and Faecalibacterium prausnitzii) and the subsequent increment of pro-inflammatory GM species (Proteobacteria), observed in the elderly, has shown a strong correlation with plasmatic IL-6 and IL-8 cytokines levels (17). These findings explain the contribution of GM to frailty development during ageing, thus suggesting its crucial involvement in several inflammatory diseases, included OA (17).

Obesity is a clear risk factor for OA onset in weight-bearing joints, but also in non-weight-bearing districts, such as wrist and hand, a higher prevalence of OA has been reported in obese patients (5). Interestingly, in obese patients with symptomatic hip or knee OA undergoing bariatric surgery, the massive post-operative weight loss is associated with an improvement of joint pain and function, together with a decreased inflammatory status.

The main determinants involved in this complex interplay might be the obesity-related GM composition changes, the impaired gut mucosal integrity observed in obese patients and the subsequent GM-derived wall components -i.e. lipopolysaccharides (LPS), peptidoglycan (PGN)- and lipid metabolites -i.e. free fatty acids (FFAs) and short-chain fatty acids (SCFAs) translocation (18). SCFAs and FFAs translocation may explain some metabolic dysregulations observed in obese patients. LPS and PGN translocation, on the other hand, might promote the OA onset by activating synovial native immunity, thus enhancing synovial inflammation and suppressing cartilage matrix synthesis, via an

up-regulation of IL-1beta (18).

Hence, LPS or LPS-binding protein (LBP) serum levels might be useful biomarkers to assess OA progression. Huang et al. in a pilot study recruiting 25 patients, have recently reported a positive association between LPS serum levels and knee OA radiographic severity, total WOMAC score, self-reported knee pain score and number of synovial activated macrophages (19).

A correct lifestyle including regular non-weight-bearing physical activity seems to have both preventive and therapeutic benefits for individuals with OA. Therefore, physical activity, besides allowing muscle mass maintenance and bone physiologic turn-over, may also positively modulate GM (20). The main elements involved in this positive modulation include immune system regulation, hormones and myokines production, enhanced gut mobility and an improved metabolism and energy expenditure (21). The GM, on its turn, in response to regular exercise, may change its composition -improving the Bacteroidetes/Firmicutes ratio-, control the oxidative stress, reduce inflammatory responses and modify of the bile acids profile (21).

Post-menopausal women generally refer with more symptomatic hip and knee OA, compared to men, but the effect of gender on gut microbiome appears still unclear (5). The study of the interactions between estrogens and GM composition has provided interesting results in hypogonadal mice models (5), but further studies are needed to better understand the clinical relevance of these findings.

Synovial fluid omics analysis in OA

Synovial fluid (SF) is a viscous and mucinous substance produced by the synovium, a specialized connective tissue that lines diarthrodial joints. Although the main function of SF is to lubricate joints, thus reducing friction between the articular surfaces, it also allows the nutrients and catabolites circulation between the avascular articular cartilage and the vascularized synovial membrane (6, 22, 23).

In recent years, the omics study of SF is gaining an increasing interest in orthopaedics and rheumatology, to better investigate the pathogenesis and improve the management of several joint diseases, including osteoarthritis (OA) (6).

Metabolomic analysis of SF in OA has recently identified some lipidic biomarkers, i.e. myristic acid, oleic acid and lanosterol, that showed a positive correlation with radiographic OA progression and structural joint deterioration (6). Moreover, a pathologic accumulation of ectopic lipids was observed in osteoarthritic, thus supporting the role of the obesity-gut dysbiosis-joint axis in the OA onset.

Interestingly, elevated LPS synovial fluid concentration levels were also observed in obese patients with concomitant knee OA (19)a key pro-inflammatory product of the microbiome, with severity of inflammation, symptoms and radiographic abnormalities of knee osteoarthritis (OA. Furthermore, synovial fibroblasts obtained from patients with OA showed a PGN-induced expression of matrix metalloproteases and proinflammatory cytokines in several in vitro studies, thus confirming the role of PGN in OA onset and progression.

Finally, two recent studies (24, 25)although the underlying mechanism remains unclear. To characterize potential SF bacterial nucleic acids, we used 16S rRNA gene amplicon sequencing to assess bacterial nucleic acid communities in 15 synovial tissue (ST have independently shown the presence of bacterial nucleic acids in the SF of patients undergoing total knee arthroplasty for OA or rheumatoid arthritis. Further studies are needed, however, to better understand the native joint microbiome's role in OA onset and progression.

How to address the gut-joint axis

Although there is increasing evidence supporting the role of GM in the pathogenesis and progression of OA, few studies have proposed effective interventions to prevent OA progression.

Based on the reported findings, effort should make in clinical practice to maintain a physiologic GM. Consequently, correct eating habits, adequate vitamins and minerals uptake, regular physical activity, avoidance of smoking and excessive energy intake, prevention of obesity, refusal of inappropriate antibiotic therapies, should be highly encouraged (5).

Furthermore, the correct use of probiotics could be also useful, since they act by modifying GM

composition, intestinal and immune system, thus giving systemic benefits to the host. Consequently, probiotic supplementation, together with a correct lifestyle, may be useful in preventing OA onset or limiting its progression.

This hypothesis should be critically assessed in randomized controlled clinical studies, in the future. These studies may lay the groundwork for precision medicine in the treatment of OA.

Future developments

This study investigated the role of gut dysbiosis and chronic low-grade inflammation in the OA onset and progression, summarizing the current evidence on this topic. In recent years, the definition of OA phenotype, the metabolic OA, has been introduced to emphasize the role of metabolic diseases and chronic inflammatory status in its pathogenesis. Several OA risk-factors, including ageing, gender, obesity and sedentary lifestyle are linked to gut dysbiosis, thus could play a relevant role in the OA progression.

Therefore, the promotion of a healthy lifestyle aiming to maintain microbiota homeostasis could be useful in slowing down OA progression. Conversely, in the future, microbiota profiling could help predict OA progression and, at the same time, the gut microbiota could be a potential target in the treatment and prevention of OA.

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Giant cell tumor of extremities, surgical treatment and local adjuvants: which is the most effective?

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Giant cell tumour (GCT) represents 5% of all primitive bone tumours. Standard surgical treatment of GCT includes intralesional excision or segmental resection. Curettage has a higher recurrence rate (10-25% in stage 2 or 3 but does preserve adjacent joint function. The use of local adjuvants such as phenol, alcohol, H_2O_2, Argon or cement may decrease recurrence rate, yet which local adjuvant works best is still, to this day, controversial. A series of 109 patients with GCT of the extremity, surgical treated in a single Institution from 2016 to 2018, were analysed in a retrospective study. The purpose of our study was to report the incidence of recurrence rate in patients with GCT of limbs treated in a single institution with different local adjuvants. The results of the present study suggests that curettage in association to cryoablation seems to reduce the recurrence rate compared to "classic" local adjuvants.

Giant cell tumour (GCT) is a primary intramedullary bone tumour. The age most concerning is from 20 to 40. The tumour often presented in epiphysis of long bones such as distal femur o radius and proximal tibia or homerus. Histologically tumour is composed of mononuclear and giant mononuclear cells similar to osteoclasts, with a variable and unpredictable growth potential (1). Surgical treatment options include intralesional excision or segmental resection. Curettage has a higher recurrence rate (20-25% in stage 2 or 3) according to Enneking (2) but does preserve adjacent joint function. The use of local adjuvants such as phenol, alcohol, H_2O_2, Argon or cement may decrease recurrence rate, but which local adjuvant works best is still, to this day, controversial.

The use of cement has more advantages: it provides structural support and prevents collapse of the cavity performed in surgery, with its exothermic reaction operates as a local adjuvant, and finally being radiopaque to the imaging, it allows an early diagnosis of any local recurrence. Although resection with wide margins minimizes tumour recurrence, it is associated with worse functional results, and usually with some possible complications in reconstruction (3). The purpose of our study was to report the incidence of recurrence rate in patients with GCT of limbs treated in a single institution with different local adjuvants. The hypothesis is that intralesional excision with cement and cryoablation as local adjuvants provides better local control compared to "classic" local adjuvants.

MATERIALS AND METHODS

We retrospectively reviewed patients treated with

Key words: Giant Cell Tumours (GCT), excision, Adjuvants, recurrence, Cryoablation

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intralesional excision for bone Giant Cell Tumours (GCT) from January 2016 and December 2018 at our Operative Unit (OU). All the patient's data were recovered from our unit clinical and radiological database. Patients were than divided into two groups: 1) Group G1 treated with curettage, "classic" local adjuvants (such as phenol, ethyl alcohol, hydrogen peroxide) and filling with cement; 2) Group G2 treated with curettage, local cryoablation and filling with cement. To reduce the risk of wound healing problems related to use of cryoablation, we protected the skin and soft tissue during the treatment of cryoablation using sterile bags of hot water (sterile gloves filled with sterile hot water). After surgical treatment, patients were clinically and radiologically followed-up at 1 month, 3 months, 6 months, 1 year and than annually.

In 5 cases a neo-adjuvant therapy with denosumab was used. We chose this treatment for GCT with a diameter > 10 cm and a distance from articular margins (articular cartilage < 2 cm). Denosumab was subcutaneously administered. We used a dose of 120 mg every 4 weeks for 4 pre-operative administrations. No post-operative administrations were used.

Our study primary endpoint was the comparison of the recurrence rate between the two groups. Statistical analysis was performed using the Statistical Package for Social Sciences, Version 13 (SPSS Inc, Chicago, Illinois). Continuous variables were showed as mean +- standard deviation (SD) and discrete variables were expressed as frequency percentages. The chi-squared test with Yates correction for small samples was used to compare discrete quantitative variables. The level of significance adopted was 5% with a 95% confidence interval.

SITE OF TUMORS	No. of cases (%)
Proximal Femur	14 (14%)
Distal Femur	19 (18%)
Proximal Tibia	23 (22%)
Distal Tibia	2 (2%)
Humerus	7 (7%)
Radius	15 (15%)
Pelvis	6 (6%)
Lumbar Vertebrae	2 (2%)
Hand	9 (9%)
Foot	5 (5%)
TOTAL	102 (100%)

Table I. *Tumour site distribution.*

Table II. *Tumor grade distribution (Campanacci classification).*

	No. of cases (%)
GRADE I	23 (23%)
GRADE II	44 (43%)
GRADE III	35 (34%)

RESULTS

Between January 2016 and December 2018, we treated 102 cases of bone GCT with intralesional excision. There were 45 patients placed into the G1 Group and 57 patients were placed into the G2 Group. Tumour site distribution is showed in the Table I.

The most involved sites were proximal tibia (22%), distal femur (19%) and radius (15%). The tumour grade (established using the Campanacci's classification) distribution is showed in the Table II.

In one case, the GCT diagnosis was made because humerus pathological fracture; this patient was treated with curettage, cryoablation and filling with cement (G2 Group). The tumour size at the moment of diagnosis was showed in Table III.

Only a minimal part of large tumours were treated with intralesional excision at our clinic. Most of them were instead treated with bone resections. Mean follow-up period was 36.4±14.3 months for the G1 Group and 39.7±20.2 months for the G2 Group. The recurrence rates were 18% into the G1 Group and 11% into the G2 Group; this difference was appreciable, but the chi-squared test analysis showed it was just not statistically significant (p =

0.44). No metastatic locations were diagnosed at the moment of the recurrence check-up. All patients with recurrence underwent revision surgery following the same protocol as the primary surgery. We found no significant differences in recurrence-free survival at 1 and 3 years after surgery between the two groups (Table IV).

No significant difference also in term of mean time recurrence detection was found (Table IV). We reported 2 cases of post-operative pathological fracture into the G2 Group; all these cases were conservatively treated and finally healed. In 6 patients from the G2 group (10.5%) we reported delayed wound healing that was completely solved in all cases with multiple superficial curettage and oral antibiotic therapy. Only one patient pre-operatively treated with denosumab and included into the G1 group showed a disease recurrence during our follow-up period.

Table III. *Tumors size at initial diagnosis.*

	No. of cases (%)
< 5 cm	81 (79%)
> 5 cm	21 (21%)

Table IV. *Comparison between G1 and G2 groups.*

	G1 Group	G2 Group
Patient's Number	45	57
Age at Initial Treatment (SD)	36,3 (12,5)	34,9 (9,9)
Sex Ratio (M/F)	24/21	32/25
Denosumab Neo-Adiuvant Therapy	3	2
Duration of follow-up (months)	36,4 ± 14,3	39,7 ± 20,2
Recidives Number	8 (18%)	6 (11%)
1-Years Recurrence Free Survival	0,96	0,98
3-Years Recurrence Free Survival	0,82	0,89
Mean Time Recurrence Detection (months)	15,5 ± 11,1	18,3 ± 14,4
Pathological Fractures	0	2

DISCUSSION

GCT is a benign bone tumour but it can have a variable course, sometimes it can remain latent (stage 1). Most of the time, however, it appears as an active lesion (stage 2) and, more rarely, as an aggressive lesion (stage 3). Exceptionally it can occur in multiple bones at same time (multicenter GCT). Despite being a benign tumor, less than 2% of patients can develop lung metastases (4), in fact these metastases mostly have a benign trend, remaining unchanged over time or even disappearing in the follow up (4). Malignant degeneration of GCT in giant cell sarcoma is very rare. Malignant degeneration can occur spontaneously (a very rare event) or following treatment with radiotherapy (most frequent event) (4).

The gold standard treatment is intralesional excision to obtain a good local control of disease and maintain a good function (1, 5-9). The recommended curettage technique involves the opening of the bone through a large cortical window that allows visualization of the entire tumour cavity (10-12). After curettage is achieved, the cavity is deepened with the use of high-speed burrs (10, 12) (Fig. 1). Some authors (5, 11, 13-15) recommend the use of local adjuvants combined with curettage to reduce the risk of recurrence. Adjuvants presumably remove the tumour cells that remain after curettage because of their thermal (liquid nitrogen, methylmethacrylate, cryoablation) or chemical (phenol, hydrogen-peroxide, ethylic alcohol) effects. After tumour evacuation, the cavity can be left unfilled or it can be filled with cement or bone grafting (6, 10, 11, 16).

Studies have found the recurrence rate as high as 50% for intralesional curettage without a local adjuvant, three-quarters within two years. To reduce the recurrence rate, local adjuvants have been used in combination with surgery. Retrospective studies have demonstrated reduced recurrence rates ranging from 13 to 22% (13). However, there are no studies in literature that unequivocally determine that one local adjuvant is better than another. Errani in all had a recurrence rate in 16% in patients having curettage, but it was lower after a curettage and the association of phenol, ethylic alcohol and cement (12.5%) (17).

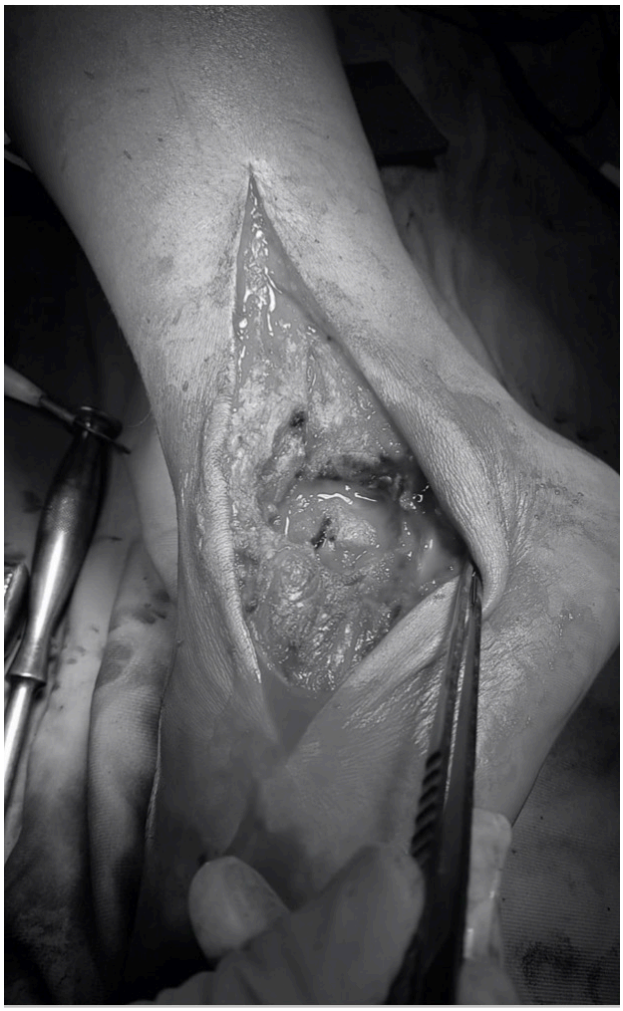

Fig. 1. *Giant Cell Tumor (GCT) of the talus after surgical curettage.*

Georg W, Omlor A, recommended the use of hydrogen peroxide to reduce the risk for tumor recurrence (18). Recently, the cryoablation was tested as a local adjuvant in GCT treatment and proved to be effective (3, 19). Cryoablation is a local treatment that induces coagulation necrosis by means of rapid freezing, slow thawing, and repetition of the freeze-thaw cycle (20) (Fig. 2, Fig. 3).

In addition, it has been recently evaluated that the possibility of using Denosumab as neo-adjuvant therapy for GCT to decrease their size or extent before surgical treatment. In fact, after treatment with denosumab there is formation of a peripheral rim of bone around the lesions and consequently make it possible to perform curettage rather than a segmental resection (21). However, Campanacci et al. reported that the use of Denosumab as neo-adjuvant therapy may determine an increasing risk of local recurrence because interfere with the ability of surgeons to extend the margins (22). The same conclusion is recently confirmed by a systematic review of the literature and meta-analysis of Xi Chen at All (23).

The results of our study, with the limits derived from the small number of cases considered, seems to suggest the cryoablation may reduce the recurrence rate compared to "classic" local adjuvants. The recurrence rates were 18% into the G1 Group and 11% into the G2 Group; this difference was appreciable, but the chi-squared test analysis showed it was just not statistically significant ($p = 0.44$).

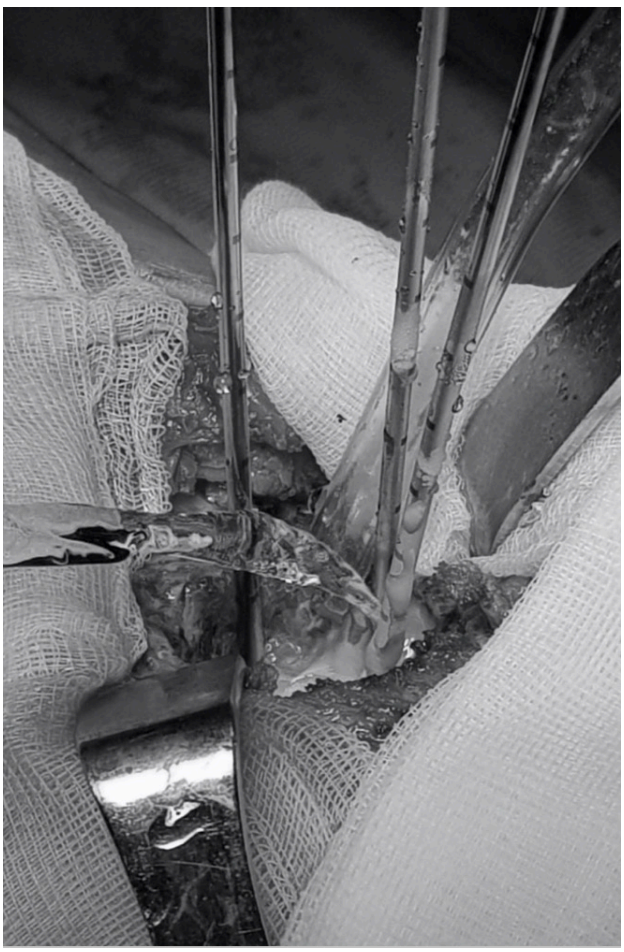

Fig. 2. *Probes positioning into the bone cavity after TCG curettage.*

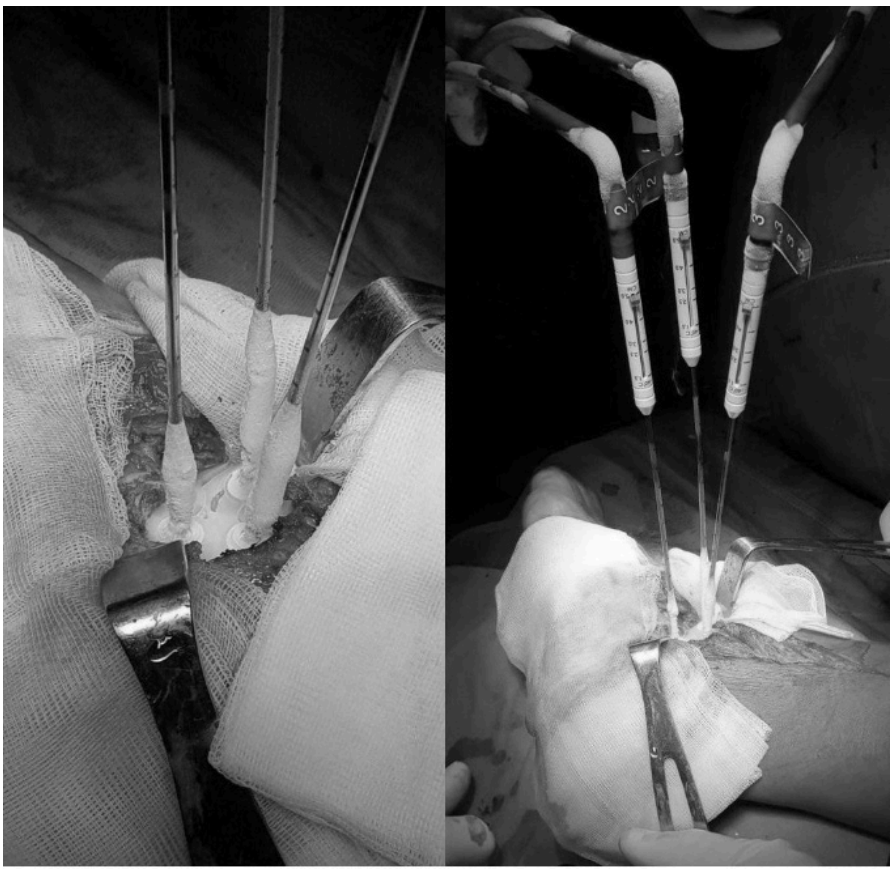

Fig. 3. *Cryotherapy application after TCG curettage.*

Our study results also highlighted the cryoablation safety. In fact - using some particular measures (such as protecting the skin with sterile gloves filled with sterile hot water and filling the residual cavity with cement) – we reported only 6 cases of delayed wound healing and 2 cases of post-operative fractures into the G2 group. All these complications completely solved without further surgical procedures.

In conclusion the present study, with the limits derived from the small number of included cases, confirmed the initial hypothesis that intralesional excision with cryoablation as local adjuvants and filling with cement may provide better local control compared to "classic" local adjuvants. To reduce the risk of fracture post cryoablation as a precaution we recommend performing an internal fixation with plate and screws. A study with a larger amount of cases will have to be performed to reach statistical significance from our results.

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Monitoring of C-reactive protein level (CRP) and Erythrocyte sedimentation rate (ESR) after total hip and knee arthroplasty

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C-reactive protein (CRP) and Erythrocyte sedimentation rate (ESR) are the two most commonly serum biomarkers for the diagnosis of periprosthetic joint infections (PJI). We monitored CRP and ESR in 60 patients affected by osteoarthritis who underwent primary total hip or knee arthroplasty to verify their utility for an early diagnosis of periprosthetic hip and knee infections. In all but two patients, both CRP and ESR increased rapidly after surgery, reaching a peak value around the 3rd day postoperatively; CRP decreased rapidly in 20 days, reaching normal value one month after surgery, while ESR decreased slowly, reaching the normal value after three months. In two patients, CRP and ESR were still elevated six months after the surgical procedure and in both cases a diagnosis of PJI was made. Our study confirms that postoperative screening of CRP and ESR values are very useful in making an early diagnosis of this serious complication.

Periprosthetic joint infection (PJI) is one of the most severe complication of total hip and knee arthroplasties. The early diagnosis of PJI is very difficult, but crucial to make it within the first post-operative month, to start the correct treatment and possible avoid prosthesis removal. The analysis of systemic inflammation markers is very useful for diagnosis of potential PJI. C-reactive protein (CRP) and Erythrocyte sedimentation rate (ESR) are the two most common serum biomarkers used for infection diagnosis (1-7). CRP is an annular pentameric protein of hepatic origin, present in blood plasma, whose circulating concentration rises in response to inflammation and it is widely used as an inflammation marker.

In the adult population, the normal concentration of CPR varies between 0.8 mg/L to 3,0 mg/L. However, some healthy adults show elevated CRP at 10 mg/L. ESR test measures the rate at which the erythrocytes, in a sample of blood, settle at the bottom. The normal reference range for ESR results is 1-13 mm/hr for males and 1-20 mm/hr for females. Both CRP and ESR values tend to rise with age, but they are always modified in the postoperative period of surgically treated patients, such as total hip or knee arthroplasties, due to surgical trauma. However, their values return to the preoperative level in uncomplicated total joint arthroplasties. The aim of the present study was to monitor the value of CPR and ESR in a series of 60 patients affected by osteoarthritis surgically treated by primary total hip or knee arthroplasty, in order to verify the utility of this blood laboratory markers, usually used for the diagnosis of infection.

MATERIALS AND METHODS

We prospectively evaluated 60 patients affected by hip or knee osteoarthritis who underwent a total joint arthroplasty. We excluded 18 patients because of systemic

Key words: C-reactive protein (CRP), Erythrocyte sedimentation rate (ESR), periprosthetic joint infection (PJI)

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inflammatory conditions, which affected the baseline values of CRP and ESR. Thirty-four patients (18 males and 16 females) were operated on by primary total hip arthroplasty, with a mean age at surgery of 68.8 years (range 51 to 82 years). Twenty-six patients (4 males and 22 females) were operated on by primary total knee arthroplasty, with a mean age at surgery of 72.2 years (range 66 to 80 years). The total hip arthroplasties were uncemented in all cases, while all the total knee arthroplasties were cemented, without involving the patella in the prosthetic implant.

All total hip and knee prostheses were implanted with a standard surgical technique, using spinal anesthesia. A prophylactic antibiotic therapy was administered preoperatively in all patient, one hour before surgery. Time of surgery ranged from 60 minutes to 120 minutes, with an average of 75 minutes. At the end of surgical procedure, a deep drainage was applied in all patients, which was removed after 24/48 hours.

Both CRP and ESR value were monitored preoperatively amd on the 3rd, 10th, 20th, 40th, 60th, 90th day after surgery. Two more checks of these blood markers were carried out at the sixth month and one year postoperatively. For the CRP measurement, a clinical chemistry analyzer was used, while for of the ESR the Westergren technique. Statistical analysis was performed using Student's t-test, that was considered significant when p value measured < 0.05.

RESULTS

The values of CRP and ESR observed in total hip and knee arthroplasties from before surgery up to a year after surgery are reported in two graphs. The data concerning the two patients in which we observed a PJI are excluded from the graphs (Fig.1 and 2).

The preoperative values of CRP in all patients but two (complicated by PJI), averaged 5±4 mg/L. After surgery, peak values were observed on the third day in both groups (104 mg/L in total hip arthroplasty and 168 mg/L in total knee arthroplasty). The difference between the postoperative peak value observed in total hip and knee arthroplasty was statistically significant. In all patients, CRP levels decreased rapidly from the 3rd to 20th day and continued to decrease more slowly, until reaching almost normal values one month after surgery (8 mg/L in total hip arthroplasty and 16 mg/L in total knee arthroplasty). CRP values returned to normal three months after surgery (Fig. 1, 2). There was statistically no significant difference in the decrease of CRP values between the two groups.

The preoperative values of ESR in all patients but two (complicated by PJI), averaged 18.5±2.5 mm/h. After surgery, a peak value was observed the third day in both groups (76 mm/h in total hip prostheses and 104 mm/h in total knee prosthesis), without any

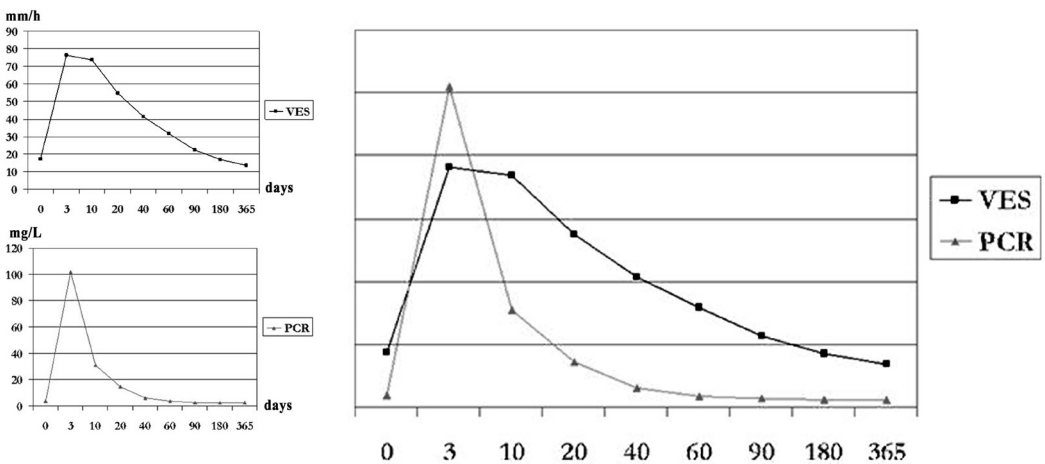

Fig. 1. *CRP and ESR trend values in a series of patients operated on by total hip arthroplasty from preoperative to one year after surgery.*

statistical difference between the two groups. In all patients operated on by total hip arthroplasty, ESR values decreased slowly from the 3rd to the 10th postoperative day, then more rapidly but steadily, reaching almost normal values after 3 months and normal values after 6 months. In all patients operated on by total knee arthroplasty, ESR values decreased steadily from the 3rd day to 3rd month after surgery. ESR values, similarly to those of the total hip arthroplasty, became almost normal after 3 months and normal after 6 months (Fig. 1, 2). There was a statistically significant difference in the decrease of ESR values between the two groups.

In two patients (one operated on by total hip prosthesis and another one by total knee prosthesis) we observed the same peak values of CRP and ESR after surgery, without observing the progressive decrease up to normalization seen in the other patients. In both cases, according to the Parvizi et al criteria (8), we diagnosed a chronic periprosthetic joint infection, which were successfully treated surgically by two stage revision.

DISCUSSION

Periprosthetic joint infection (PJI) rate is about 2% in spite of the advancement in implant design, the more accurate perioperative protocols and the better surgical procedures (9-10). It's well known that postoperative infections represent a severe complication, difficult to eradicate, in orthopaedic practice, in adults and during skeletal grwth (11-18). PJI represents one of the most serious complications following total joint replacement and an early and accurate diagnosis is very important for successful management. The diagnosis of PJI, especially in the early postoperative days, may be particularly difficult to make due to its non-specific presentation and the analysis of serum biomarkers is helpful for an early diagnosis and treatment.

C-reactive protein (CRP) and Erythrocyte sedimentation rate (ESR) are the two most common serum biomarkers used for diagnosis of potential PJI and play an important role in determining the presence of an early postoperative infection after total joint replacement (4-6, 19). Other serum biomarkers, as interleukin-6 (IL-6), procalcitonin (PCT) and D-dimer, are useful, but have not replaced CRP and ESR as the first-line screening tests (6, 20-22).

Some authors have reported the monitoring of CRP and ESR during the first weeks after total joint replacement with the aim to early detect PJI. White et al. (2) measured CRP level after total hip and total knee replacement. The peak was observed on the

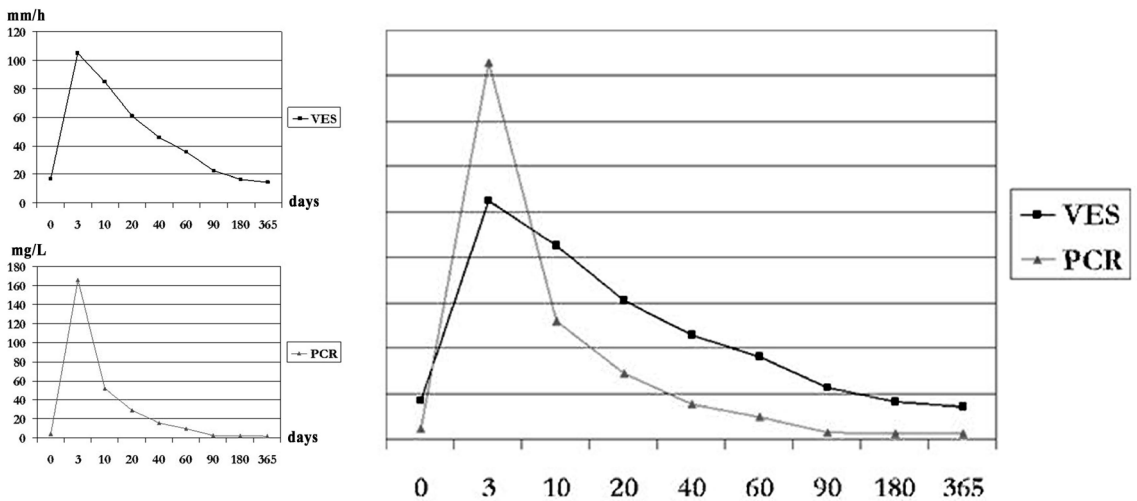

Fig. 2. *CRP and ESR trend values in a series of patients operated on by total knee arthroplasty from preoperative to one year after surgery.*

2nd and 3rd postoperative day for both groups. The level of CRP after total knee arthroplasty was higher than that observed after total hip arthroplasty, until the 7th day after surgery, when CRP levels decreased at a similar rate in both groups. They conclude that any upward trend of the CRP level after the 3rd postoperative day may suggest infection.

Other authors (3), reported a study on 28 patients measuring the CRP and ESR values after total hip and knee arthroplasty. They reported that in both groups CRP level increased rapidly after surgery peaking on day 2 and dropping gradually to preoperative values on day 21 in total hip replacement and at the end of the second month after surgery in total knee replacement. ESR peaked on day 5 after the operation and dropped close to preoperative values at the end of the third month in total hip replacement patients, but at the end of the ninth month after surgery in total knee arthroplasty patients.

In this last group, ESR remained slightly above the preoperative values even one year after surgery. Moreschini et al (23), in a report on postoperative physiopathological analysis of inflammatory parameters in patients undergoing hip or knee arthroplasty concluded that the CRP values >1mg/dl coupled with ESR values more than three times higher than the basal levels, persisting for more than 60 days after surgery, justified suspicion of infection.

Park et al. (24) in a prospective study, evaluated 228 consecutive patients with osteoarthritis who underwent primary total knee arthroplasty (336 knees). CRP level increased rapidly, reaching a peak on the 2nd day and returned to preoperative values on the 90th day after surgery. ESR level peaked on the 5th day and returned close to the preoperative values, like the CRP, on the 90th day after the operation. The authors reported wide variations of serum biomarkers and in many cases the typical pattern was not present. However, they concluded that their findings should help orthopaedic surgeons determine the presence of PJI of the knee.

More recently, other authors (19) reported a study on 51 patients operated on by total hip replacement for primary osteoarthritis, in which the serum CRP level showed a marked increase on the 3rd postoperative day and returned close to preoperative values at the end of the first month after surgery. The authors concluded that CRP is very sensitive to inflammation. In another recent study (25), on CRP and ESR values measured after total hip and knee replacements, the authors reported that the level of CRP reached its maximum value on the 2nd day, but in some cases did not reach the normal value after one year. ESR values had an upward trend during the first 5 days and then decreased but, in some cases, like with the CRP, did not reach its preoperative value after one year. The authors concluded that patients with a persistent high value of CPR and/or ESR, especially symptomatic ones, must be further investigated in order to exclude a PJI. Maniar et al (21) studied serum IL-6 and CRP levels in 50 patients undergoing primary total knee arthroplasty at five time points: 12 hours preoperatively and postoperatively at 12 hours, 48 hours, 4 days and 2 weeks. They concluded that CRP showed a gradual rise to peak in 48 hours then fell gradually, remaining elevated at 4 days and higher than baseline level at two weeks.

To the best of our knowledge, in all papers that reported the trend of CRP and ESR from the preoperative period to 90-180 days postoperatively after total jpint replacement, the level of these biomarkers rapidly increased and reached a peak in few days. This was followed by a gradual decrease, with different trends, with a return to baseline values between one to six months. In most of the cases, CRP returned to normal more rapidly compared to ESR. Our results confirm these data. However, we observed, in agreement with other authors (2, 3), a statistically significant difference between the patients surgically treated by hip and knee total prosthesis. In fact, in our series of patients, the mean peak value of CRP was more than one third higher in patients with total knee prosthesis, despite a similar trend. We speculate that the two main reasons for higher CRP values in total knee replacements may be the use of tourniquet and cement.

Concerning the difference between CRP and ESR levels, we agree with other authors that CRP is more reliable, since it returns to the baseline level at around one month. Moreover, in some patients, especially during old age, ESR may remain higher

for new occurred and other systemic inflammatory conditions, reducing the reliability of this marker.

In conclusion, according to the previous reported studies, we believe that a persistent high value of CRP and ESR may suggest a PJI, and further diagnostic investigations are necessary. To confirm this, in our series, in the only two patients with persistent high level of both biomarkers, we had a definite diagnosis of a PJI.

ACKNOWLEDGEMENTS

This study was approved by the ethics committee of Policlinico Tor Vergata, Rome; the patients were informed about the study and consented to participate. The data of the manuscript is available. The authors declare that there is no conflict of interest regarding the publication of this paper. No funds have been received by the authors.

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Orthopaedic aspects in seventy-two children affected by Marfan syndrome. Correlations between pathological features and fibrillin-1 gene mutations

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Marfan syndrome is an autosomal dominant disorder of the connective tissue caused by mutations of the fibrillin-1 gene (FBN1) that primarly involves the cardiovascular, skeletal and ocular systems. We investigated 72 children affected by Marfan syndrome in order to identify possible correlations between some musculoskeletal features and specific mutations of fibrillin-1 gene. The following FBN-1 gene mutations have been observed: a missense mutation in 21 children, a stop mutation in 9, a splice mutation in 15 and other mutations in the remaining 27 patients. We observed a statistical significant association between chest asymmetry and splice mutation (p=0.031) and between scoliosis >20° or thoracolumbar kyphosis and stop mutation (p=0.039). However, we did not find a true genotype-phenotype correlation between the fibrillin-1 gene mutations observed and the prognosis of the disease. Future studies are necessary to demonstrate further genotype-phenotype correlations in order to identify early prognostic markers of Marfan syndrome and to plan the most appropriate clinical management accordingly.

Marfan syndrome is an autosomal dominant disorder of the connective tissue caused by fibrillin-1 (FBN1) gene mutations located on chromosome 15 (1, 2). The FBN1 gene codifies fibrillin-1, which represents an important structural component of the extracellular matrix that contributes to the integrity of elastic fibres of the connective tissue (3). The diagnosis of Marfan syndrome is defined by the Ghent criteria (4, 5).

In Marfan syndrome, aortic root dilatation can lead to thoracic aorta aneurysm or to aortic dissection which are the main cause of morbidity and mortality. Other important signs of Marfan syndrome are musculoskeletal abnormalities, ectopia lentis and dural ectasia, all of which have a wide phenotypic variability. Regarding the musculoskeletal abnormalities, the most frequent deformities involves the chest wall and the spine. On the contrary, developmental dysplasia of the hip (6) and congenital clubfoot (7, 8), which represent the most common congenital deformities in children, have never been reported. A definitive diagnosis of this disorder cannot always be reached only by the clinical criteria, especially in children, since there is a great variability in terms of age of onset, severity and number of pathological signs.

Therefore, the knowledge of the genetic aspects is very important for an early diagnosis (9-13). Recently some authors have suggested that the type of FBN1 mutation could be correlated to the prognosis of the disease (12, 14).

In the present study we report the results of a genetic analysis performed in 72 pediatric patients with a diagnosis of Marfan syndrome. The aim of our study was to investigate possible association between specific mutations observed in the fibrillin-1 gene and some musculoskeletal features, and its possible implication in the prognosis and the management of the disease.

Key words: Marfan syndrome, FBN1, chest asymmetry

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MATERIALS AND METHODS

We investigated 72 children (mean age 7.9±5 years; 35 male and 37 female) with a diagnosis of Marfan Syndrome, admitted at the referral center of rare disease of our Hospital. This study was approved by the ethics committee of our hospital; the patients' parents were informed and consented to participate to the study.

All children were evaluated by a team that included a cardiologist, a cardiac surgeon, an orthopaedic surgeon, an ophthalmologist, an odontologist and a pediatrician. A blood test was performed in all children for genetic assessment in order to determinate the FBN1 mutations. Orthopaedic examination was performed to search for the typical skeletal aspects of the disease that are: "wrist sign", "thumb sign", pectus carinatum or excavatum, chest asymmetry, hindfoot deformity, flatfoot, reduced elbow extension, reduced upper segment to lower segment (US/LS) ratio, acetabular protrusion, scoliosis or kyphosis. We asked patients' parents if the children had a myopia greater than 3 diopters. In all children we also evaluated the typical facial features as dolichocephaly, frontal bossing, deeply sunken eyes and high arch palate and the skin striae.

At the genetic examination the FBN1 gene extracted from peripheral blood was analyzed. The mutations of FBN1 gene were grouped as follows: missense, stop, splice and other. A statistical analysis was performed according to the following criteria. All quantitative variables were synthesized as a mean and standard deviation and categorical variables such as frequencies and percentages. The Fisher test or the X2 test were calculated for qualitative variables. An one-way ANOVA model was used for comparison between systemic score and genetic mutations. The analyses were processed using the STATA 13.1 software and all tests were considered significant for relative values $p < 0.05$.

RESULTS

The diagnosis of Marfan syndrome was confirmed in all 72 children. We found a missense mutation in 21 children (29.2%), a stop mutation in 9 (12.5%), a splice mutation in 15 (20.8%) and other mutations in the remaining 27 cases (37.5%). We have statistically linked the single fibrillin-1 gene mutations previously reported with the clinical aspects observed in all children (musculoskeletal features, myopathy, facial features and skin striae) (Table I). We observed a statistical significant association between splice mutation and chest asymmetry (p= 0.031) and between stop mutation and scoliosis >20° or thoracolumbar kyphosis (p=0.039). On the contrary, we did not observe any statistical association between the fibrillin-1 mutations reported and the other clinical signs observed in all children (Table I).

Chest asymmetry was present in 11 children and in 6 of these a splice mutation was present; this deformity is caused by a complex asymmetry of the chest wall with possible mild or moderate clinical sign of pectus excavatum or carinatum and spinal deformities.

Table I. *Correlation between musculoskeletal features and fibrillin-1 gene mutations.*

Variables	Missense (n=21)		STOP (n=9)		Splice (N=15)		Other (N=27)		
	N. patients	%	N. patients	%	N. patients	%	N. patients	%	p-value
Wrist sign	12	57.1	7	77.8	8	53.3	14	51.9	0.614
Thumb sign	12	57.1	6	66.7	6	40.0	12	44.4	0.515
Pectus carinatum deformity	7	33.3	4	44.4	5	33.3	7	25.9	0.744
Pectus excavatum	2	9.5	3	33.3	1	6.7	6	22.2	0.254
Chest asymmetry	3	14.3	0	0.0	6	40.0	2	7.4	0.031
Hindfoot deformity	6	28.6	4	44.4	8	53.3	11	40.7	0.495
Flatfoot	8	38.1	4	44.4	8	53.3	14	51.9	0.783
Reduced elbow extension	1	4.8	0	0.0	1	6.7	1	3.7	1.000
Upper segment/lower segment ratio	4	19.0	2	22.2	2	13.3	10	37.0	0.315
Acetabular protrusion	3	14.3	0	0.0	0	0.0	1	3.7	0.308
Scoliosis > 20° or thoracolumbar Kyphosis	6	28.6	5	55.6	1	6.7	11	40.7	0.039
Myopathy > 3 Diopter	3	14.3	1	11.1	1	6.7	1	3.7	0.539
Facial features	2	9.5	1	11.1	1	6.7	5	18.5	0.765
Skin Striae	0	0.0	0	0.0	2	13.3	0	0.0	0.055

ƒ = Fisher test

In 12 children we observed a pectus excavatum and in 23 a pectus carinatum but all these deformities were mild or moderate. However, we did not find any statistical association with the fibrillin-gene mutation (pectus excavatum: p= 0.254) (pectus carinatum: p= 0.744).

Scoliosis or thoracolumbar kyphosis were observed in 23 patients, and in 5 of these we found a stop mutation. The scoliosis, measured according to the Cobb method, ranged from 24 to 36 degrees, while the thoracolumbar kyphosis, ranged from 44 to 58 degrees. No patient with chest or spinal deformities had significant pain, respiratory disfunction or other clinical symptoms. Six children with spinal deformities were treated with a brace.

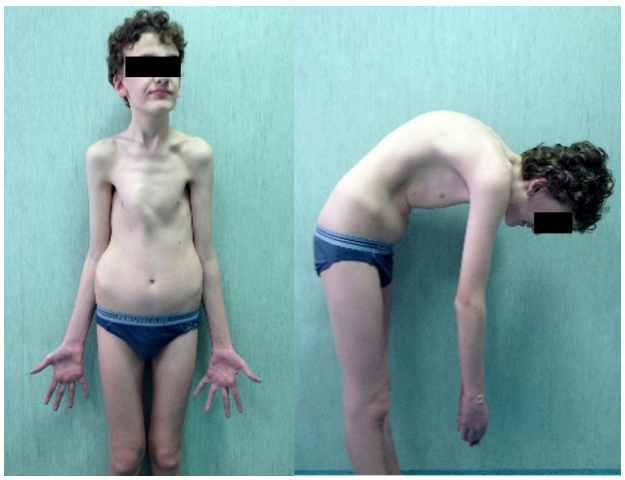

Fig.1. *Eleven-year old boy affected by Marfan syndrome with a complex chest asymmetry and associated thoracic kyphosis. The genetic analysis showed a splice mutation of the FBN1 gene.*

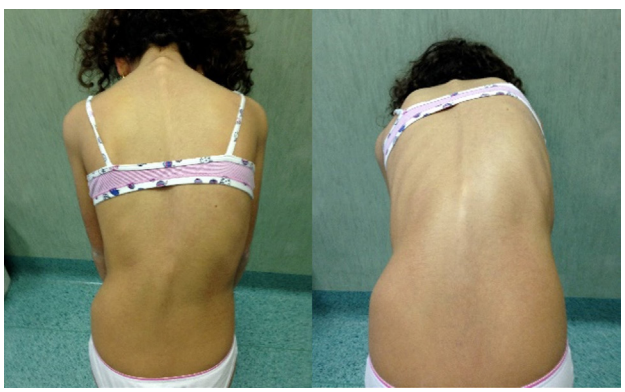

Fig. 2. *Ten-year old girl affected by Marfan syndrome with thoracolumbar scoliosis that measured 37°. The genetic analysis showed a stop mutation of the FBN1 gene.*

DISCUSSION

Marfan Syndrome is an autosomal dominant disease of connective tissue that primarly involves the cardiovascular, skeletal and ocular systems. This syndrome is caused by mutations in the fibrillin-1 gene (FBN1). Fibrillin-1 is a glycoprotein that plays a dominant role in formation and deposition of elastic fibers (1-3). Fig.1.

The diagnosis of Marfan syndrome in children may be difficult because the clinical features are often age related; in these cases genetic analysis becomes fundamental for a correct and early identification of the disease. However, genetic studies of the different types of fibrillin-1 mutation might play an important role even in the prognosis of the disease.

Reports describing chromosomal alterations involving FBN1 gene are rare and very poor genotype-phenotype correlations have been demonstrated (15, 16). Interpretations of genomic data and possible genotype-phenotype correlations are complicated by a high rate of intronic variants of unknown significance (17, 18). However, the studies on the genotype-phenotype correlations in Marfan syndrome have recently increased and some reports suggested that the type of FBN1 mutation might be related to the prognosis of the disease.

Some authors (10), in a study on 80 patients affected by Marfan syndrome, reported that a low level of residual wild-type fibrillin-1 mRNA is correlated with a high risk of ectopia lentis, pectus abnormality and tends to increase the risk of aortic dilatation. Recently, other authors (13) reported that variants affecting or creating cysteine residues are more commonly associated with ectopia lentis, while null alleles are typically correlated with an increased rate of aortic events in a younger age and thoracic deformities.

Further authors (12), in the same year, published a genetic study including 84 patients with a diagnosis of Marfan syndrome in accordance with Ghent criteria, and reported that "truncating" variants in FBN1 is related with a higher proportion of aortic events, compare to a more benign course in patients with missense mutations. They also concluded that genetic findings could have importance not only

in the diagnosis, but also in risk stratification and clinical management of these patients. More recently, another study (19) reported that nonsense, frameshift and some splicing variants appear correlated with a more severe skin and skeletal phenotype, as compared to in-frame variants.

In 11 patients of our series we observed a complex chest asymmetry with possible association of spinal deformity and in 23 patients, scoliosis >20° or thoracolumbar kyphosis. According to different mutations of the FBN1 gene our results showed a statistically significant association between splice mutation and chest asymmetry and between stop mutation and scoliosis >20° or thoracolumbar kyphosis. However we did not find a proved genotype-phenotype correlation between these fibrillin-1 gene mutations and the prognosis of these patients.

Congenital chest wall deformities are generally present as an isolated form. However in almost 6% of cases, they are associated with syndromic conditions and systemic diseases (20). Pectus excavatum is the most common chest wall deformity observed in Marfan syndrome. The main characteristic of pectus excavatum is the inward displacement of the sternum and the adiacent costal cartilages (21, 22). Pectus carinatum is the second most common deformity of chest wall reported in Marfan syndrome and it is characterized by anterior protrusion of the sternum or of the adjacent ribs or both. However, in our reported cases we observed 12 children with pectus excavatum and 23 with pectus carinatum. In these cases the deformities were mild or moderate and we did not observe cardiopulmonary impairment. The progression of these chest wall deformities, however, may be particularly evident at time of puberty and a mild defect may quickly turn into a severe one (20-22). Fig. 2.

Scoliosis or thoracolumbar kyphosis are frequent manifestations of Marfan syndrome and some authors report an incidence of these deformities in about 60% of cases (23). In our series we observed these spinal deformities in about 30% of cases, although we did not consider mild deformities (24).

In conclusion, in Marfan syndrome there is a wide clinical variability and the phenotype evolves over the patient's life course (25). We believe that further genetic studies are necessary to demonstrate other genotype-phenotype correlations that can identify the prognosis of the disease early on and allow to perform an appropriate clinical management.

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The effectiveness of telerehabilitation after hip or knee arthroplasty: a narrative review

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Telerehabilitation is defined as a set of tools, procedures, and protocols to deliver rehabilitation programs remotely. It involves the use of various communication technologies to efficiently provide rehabilitation services distantly or via some other remote environment. After an orthopedic procedure, physical rehabilitation is essential to restore joint's function, to improve quality of life as well as to relieve pain, to recovery independence. The effectiveness of telerehabilitation has been studied in literature. The aim of this narrative review is to update the current evidence, evaluate the efficacy of telerehabilitation after hip, and knee prosthesis surgery for end stage arthrosis. Results show that it is useful to integrate traditional interventions with telerehabilitation to accelerate efficiency in existing healthcare delivery systems. Future high-methodological-quality studies should be conducted to evaluate the long-term efficacy and safety of innovative technologies.

Osteoarthritis (OA) is the most common joint disease worldwide, prevalent among elderly people, which mostly affects the hip and knee (1). Total hip arthroplasty (THA) or total knee arthroplasty (TKA) are surgical interventions for end-stage symptomatic osteoarthritis; a significant increase of total number of this type of endoprosthetic interventions has been registered in most industrialized countries during the the last decades (2), and a further increase is to be expected due to the aging population and the growing rate of obesity, with a huge demand for health services. Given the large financial burden of these procedures, the search for efficient assistance strategies for THA e TKA patients are a matter of considerable public interest.

After an orthopedic procedure, physical rehabilitation is essential to restore joint's function, to improve quality of life as well as to relieve pain, to recovery independence, and should be started preoperatively, and continued for several months after hip or knee replacement, in order to achieve successful post-operative outcomes.

The effectiveness of rehabilitation after THA or TKA has been widely demonstrated in literature (3-5). Rehabilitation optimizes physical activity post-surgery and enhances functional recovery. Physiotherapeutic exercises and education have shown to be effective in reducing pain and improving physical functioning (6).

However, the downside of face-to-face interventions is that they are expensive. With the ageing of population and the limited resources allocated for public health, there is a need for cost-effective interventions to manage hip and knee postoperative clinical and functional recovery (7). The spread of low-cost Internet connection and forefront web-communication technologies in most

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countries, has allowed the application of technology-based solutions in the rehabilitation field.

Telerehabilitation is defined as a set of tools, procedures, and protocols to deliver rehabilitation programs remotely (8). It involves the use of various communication technologies to efficiently provide rehabilitation services distantly or via some other remote environment (9). Blended physiotherapy, in which physiotherapy sessions and an online guidance are integrated, might allow to carry out home-based physical therapy, reducing disease related costs and widening the access to care. This new way of delivering physiotherapy includes a wide range of services such as education, supervision, assessment, monitoring, intervention and counselling. In literature three main different types of blended physiotherapy are described:

1. Telephone-based rehabilitation, that includes rehabilitative counselling and coaching via tetephone or video-teleconferencing.
2. Game-based therapy, using video games, virtual reality, or visual biofeedback.
3. Web-based therapy, including educational softwares and interactive online training platforms. Websites and apps have the potential to partly replace face-to-face physiotherapy sessions and provide possibilities to support patients in taking an active role within their disease management. In this category are included. Exercises (10). This blended intervention consists of a web-application integrated within regular face-to-face physiotherapy sessions.

The aim of this review is to update the current evidence and evaluate the efficacy and cost-effectiveness of telerehabilitation in people undergoing TKA and THA.

MATERIALS AND METHODS

A comprehensive literature search of the MEDLINE (via PubMed), Cochrane Library, PEDro was conducted in May 2020, using the following searchMESH terms: “telerehabilitation” and “hip arthroplasty” or knee arthroplastyfor end-stage symptomatic osteoarthritisinclude reviews methanalisis and randomised controlled trials.

RESULTS

Many reliable literature sources have stated the effectiveness of telerehabilitation in improving patient satisfaction and health outcomes in variuos clinical conditions (11). In the last few decades, strong evidence in favor of telerehabilitation among patients undergoing total knee and hip arthroplasty is growing too (12). Telerehabilitation is rapidly spreading as an alternative or complimentary therapy to conventional face-to-face physiotherapeutic interventions, demonstrating its potential to maximize patient recovery in a cost-effective way. Especially after orthopedic surgery, telerehabilitation seems to be a promising offer for the recovery of motor function (12).

However, many reviews have underlined that evidence of clinical and economic effectiveness of the telerehabilitation system and blended interventions is still lacking (12, 13). Despite the increasing popularity of available technologies, only insufficient data are currently available for the effectiveness of telerehabilitation for patients after hip or knee replacement (14). This could be attributable to the quantity and the quality of telerehabilitation studies. A few systematic reviews of telerehabilitation have been conducted, but only included a handful of trials (12). Often studies lack of methodological rigor and variability (8) or remain inconclusive.

The difficulties in conducting telerehabilitation research is due to the absence of validated clinical outcomes, too small of a sample size, and a lack of comparison between groups. Moreover, differences in telerehabilitation interventions, treatment period, and follow-up, create doubts in identifying whether the telerehabilitation gives comparable or better results. A frequent problem in studies of telerehabilitation is the lack of binding therapists and patients. Telerehabilitation research is generally not very good and there are many reviews that criticize this aspect (12). There is still a poor database for telerehabilitation studies, after a musculoskeletal surgery, that provides useful data on clinical outcomes. In addition, although trials supporting the role of telerehabilitation have started to emerge in recent times, application in clinical practice results are slow (15).

As for the effectiveness of telerehabilitation, the most recent review for hip or knee arthroplasty (11) found for TKA a moderate-quality of evidence in pain reduction and low-quality of evidence for functional improvement; for THA, most of the trials had only short-term follow-ups, so results have low-quality evidence and poor clinical significance (11).

DISCUSSION

One of the cardinal points of our research is that the innovative devices or digital technologies of the telerehabilitation system, should not be viewed as a distinct therapeutic tool, but as a complement for traditional intervention, with the aim to bridge gaps or accelerate efficiency in the existing healthcare delivery system. In fact, the majority of the studies reviewed, showed that the association of telerehabilitation and usual face-to-face physical therapy was more effective than face-to-face treatment alone; in addition investigations demonstrated that tele-assisted rehabilitative interventions alone were found to be equivalent to face-to-face interventions for functional improvement and pain reduction in people with musculoskeletal conditions (12, 14). The use of telerehabilitation with patients having just undergone knee or hip replacements was equivalent to usual physiotherapy in terms of the difference achieved in the 6-minute walk test, functional mobility, health-related quality of life, and joint-related complaints (14).

The findings from another systematic review (16) provides evidence for the effectiveness of telerehabilitation therapy in patients after TKA, showing that blended physiotherapy is as effective as the conventional rehabilitation in active knee flexion and extension, improving hamstring muscle strength, reducing the swelling of knee joints, and even superior in some of the outcomes such as improving quadriceps muscle strength, and stiffness subsection of the WOMAC scale.

Few studies investigated user experience, however a satisfying adherence to telerehabilitation programs was found. Although most of the study population were older adults, a great compliance and motivation towards different types of technologies were indicated: smartphone, tablets, game consoles and Wii Fit. However some obstacles to the optimal course of telerehabilitative programs emerged, such as poor home internet connection, delayed technology installation, poor visual quality of the videoconference, technological barriers referred to cost and battery life of the devices, and poor familiarity with the technology (11). These highlighted the need for elderly user-friendly devices, continuous customer support and receiving feedback.

Regading compliance to telerehabilitation, a high rate of compliance was reported, this may be due to its accessiblity at any time and place, with a good adherence also for patients who returned to work, or to the close monitoring of the supervising physiotherapist in the rehabilitation center, who is familiar with the patients because of their previous inpatient stay, with the possibility to communicate and exchange feedbacks via text or voice messages (14). Many patients were motivated by the compilation of a training weekly diary (14).

About satisfaction, various questionnaires and surveys were used to document patient's perspective, principally addressing the technical features of using this technology and the communication pattern among the participants (16). A study evaluated the experience of both patient and physiotherapist in using the telerehabilitation therapy after TKA surgery (16), reporting that patients preferred telerehabilitation over conventional face-to-face physical therapy because the treatment was personalized using the computer system, and physiotherapists were satisfied too, due to the virtual telerehabilitation system.

From our review, some potential advantages of telerehabilitation should be mentioned. Compared to traditional rehabilitation, services delivered remotely via telephone or internet are more affordable and accessible, particularly for people living in rural areas (11).

The major advantage with the telerehabilitation therapy as reported by all patients was the elimination of transportation time for both the patient and therapist.

Telerehabilitation can be performed at any self-determined time and could enhance the adherence and compliance, especially of employed patients. Telerehabilitation could increase the access to physiotherapy, where appropriate rehabilitative

structures and servives run out.

The intervention costs of blended physiotherapy could be lower compared to usual physiotherapeutic interventions, due to the need of fewer sessions for home-based interventions compared to usual physiotherapy programs (13); so we consider the probability of e-Exercise being cost-effective compared with usual physiotherapy, although currently it has not yet been demonstrated (13).

In addition, telerehabilitation systems integrated with biosensors, accelerometers and educational software provides individualised support for people to monitor the progress of their physical rehabilitation at home, while allowing the therapist to support patients through exercise at home and intervene in a timely mannor (17). The online applications can support patients in taking an active role in the management of their chronic condition in daily living, even during the maintenance phase, thus taking an educational role.

Although no adverse effects were reported in the trials of the current review, the safety of the technology-assisted rehabilitation needs to be better understood. Dynamic or wide movements executed during games, virtual reality or videoconferencing can increase falls, risks, or other injuries (18).

The telerehabilitation systems studied until now often differ in terms of their communication structures and their feedback options. Thus, Moffet et al. (19) and Tousignant et al. (20) investigated synchronous telerehabilitation applications where the physiotherapist and the patient communicated in real-time via videoconferencing, while Bini et al. (21) and Piqueras et al. (22) studied systems in which the communication between therapist and patient took place with a time delay.

Telerehabilitation will play an important role in improving, or at least maintaining, the continuity of rehabilitation care and services. This literature review has demonstrated that the home telerehabilitation method is an acceptable method for patients who underwent knee or hip arthroplasty. It seems to be a good choice since it can be performed irrespective of location and time, and it has the potential to increase both utilization and therapy adherence. Telerehabilitation appears to be an effective alternative to face-to-face service delivery after hospital discharge of patients following total knee arthroplasty and hip replacement. Previously conducted RCTs showed comparable effects between telerehabilitation and the conventional physical therapy in short-termfollow-ups.

The most recent review (11) showed moderate- to low-quality of evidence that telerehabilitation, showed most improvements in pain and function for people following TKA, comparing to usual rehabilitation. However, the effect size was too small to be clinically significant. Future high-methodological-quality studies should be conducted to evaluate the long-term efficacy and safety of innovative technologies, especially for post-THA and TKA rehabilitation, to identify whether positive results are due to the type of intervention or the increased frequency and intensity that telerehabilitation allows.

Cost-effectiveness evaluations should also be planned alongside the clinical trial to provide a more holistic view to healthcare providers about the viability of this alternative to the conventional therapy for implementation (11).

Limitation of the study

Some limitations should be mentioned. The trials reviewed, used heterogeneous outcome measures. Consensus on a set of suitable outcome measures needs to be reached for future trials. In addition, there is insufficient long-term follow up, for giving results, scientific significance. A common risk of bias of the studies included is the lack of blinding of participants and therapists, so future research should pay attention to the methodological aspects to minimise the biases. Greater homogeneity is needed in terms of type, duration, and intervention follow-up for each specific pathology.

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Osteosarcopenia in hip fracture: taking cues from pathophysiology for clinical practice

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Hip fractures are common in older and frail adults, and the risk of adverse outcomes and mortality is significantly increased in patients affected by osteosarcopenia. Identifying particularly vulnerable subjects is a critical step to act aimed at promoting postoperative recovery and reducing the risk of adverse events. However, the diagnostic criteria that are currently used to establish the severity of osteosarcopenia are not easily applicable in patients with hip fractures and impaired mobility. In this review, the new knowledge on the pathophysiology of osteosarcopenia that provides several cues for studying biomarkers potentially useful in clinical practice is summarized. Although significant progress has been obtained in understanding the biological mechanisms leading to the involution of the bone–muscle unit, further studies are needed to identify clinically relevant biomarkers and their diagnostic accuracy in establishing the severity of the osteosarcopenia, predicting adverse outcomes, and guiding physicians in choosing appropriate therapeutic interventions.

Osteosarcopenia is a geriatric syndrome in which the low bone mineral density (BMD) and the deterioration of bone microarchitecture (osteopenia/osteoporosis) are combined with a decrease in muscle mass, strength, and functional capacity (sarcopenia) (1). Together, these diseases increase the risk of falls, fractures, and hospitalizations in older people in comparison with what is observed in patients with osteoporosis or sarcopenia alone (2). Although prevention of fragility fractures has become a public health priority and intervention strategies for the monitoring of osteoporotic patients have been long established (3), the assessment of sarcopenia has been considered only in the last decade. In a recent systematic review, the prevalence of sarcopenia in patients with hip fracture has been estimated, and the reported frequencies ranged from 17.1% to 95% (4). The heterogeneity of the data is likely caused by differences in the clinical definition of sarcopenia because various diagnostic criteria and cutoff points for anthropometric and functional measurements have been adopted by the authors (Table I).

Kirk et al. (2020) proposed an algorithm for the diagnosis and treatment of osteosarcopenia (2). First, a comprehensive medical history assessment allows identifying secondary causes of osteoporosis and sarcopenia, such as concomitant diseases, medications, nutritional status, and lifestyle (Table II). The absence of identifiable secondary causes indicates a primary, age-related osteosarcopenia.

The use of SARC-F questionnaire is proposed as initial step to find individuals with probable muscle impairment (5). Then, the diagnostic workup should proceed to confirm the diagnosis and to evaluate the severity. According to the recently revised EWGSOP criteria (6), sarcopenia is probable if SARC-F score

Key words: hip fracture, osteoporosis, sarcopenia, bone, muscle

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point is >4 and low muscle strength is detected; the diagnosis is confirmed by the presence of low muscle quantity or quality; finally, if low strength, low quantity/quality, and low physical performance are all detected, sarcopenia is considered severe (Table I).

Dual-energy X-ray absorptiometry (DXA) is the most commonly used technique to quantitatively assess BMD, and the pathological bone loss is defined on the basis of T-score values (osteopenia, <1 and >2.5; osteoporosis, <2.5). Assessment of bone quality may be additionally explored by evaluating the trabecular bone score, which reflects bone microarchitecture, or using high-resolution peripheral quantitative computed tomography (CT) that allows an accurate assessment of bone strength. Serum markers of bone resorption and bone formation are considered a useful and inexpensive tool for evaluating turnover rate (high or low) and response to any treatment. Also, laboratory testing considering renal function, calcium-phosphorus balance, other electrolytes (in particular, potassium and magnesium), and calciotropic hormones provide information on the status of the mineral metabolism.

Several technologies are currently employed to estimate muscle mass, such as DXA, magnetic resonance imaging (MRI), CT, and bioelectrical impedance analysis. Ultrasonography allows measurement of muscle quantity, identifying muscle wasting, and as a measure of muscle quality. It is widely employed as research technique, but its use has recently been expanded in clinical practice to support the diagnosis of sarcopenia in older adults. MRI, CT, and muscle biopsies have also been used to assess muscle quality and observe muscle architecture and composition (6).

The algorithm described earlier is feasible in outpatients or, more generally, in collaborating patients, but it may hardly be implemented in individuals who arrive at the orthopedic emergency department with a fragility fracture. It is probable that no information regarding the presence of osteosarcopenia is available, and, at the time of admission, the diagnostic process cannot be started because of the different priorities in terms of clinical management and the difficulty to accomplish the physical performance analyses in patients with hip fracture. However, the decline in the quantity and quality of muscle mass is expected after surgery, because of the hospitalization and the prolonged decrease in mobility. Thus, a vicious cycle is established that may affect the peri- and postoperative courses and foster the frailty condition.

In a recent systematic review, multiple predictors of poor functional outcomes and mortality have been evaluated, and grip strength and frailty were identified as significant risk factors (7). Elderly patients with hip fractures and sarcopenia require more blood transfusion volume during the perioperative period and have prolonged hospitalization after surgery (8), a poor quality of life at 6-months and 1-year after surgery (9, 10), and a higher mortality rate at 1-year (11-13), 5-year (14) and 7-year follow-up period compared with the nonsarcopenic patients (15).

Therefore, the condition of sarcopenia in patients with hip fracture should be recognized to identify particularly vulnerable subjects who could benefit from personalized treatments aimed at promoting postoperative recovery and reducing the risk of negative outcomes. In this context, the knowledge on the pathophysiology of osteosarcopenia provides several cues for finding biomarkers that could facilitate diagnosis.

Pathophysiology of osteosarcopenia

The pathogenesis of osteosarcopenia is multifactorial, with a collection of genetic, endocrine, metabolic, nutritional factors, lifestyle and concomitant diseases that contribute to the progressive physiological loss of bone and muscle mass that is expected with advancing age (16). The pathophysiology of osteosarcopenia is attributable to a main player, that is changes in the muscle and bone interaction, independently of the causes (i.e. age-related, or secondary to other factors) (Table II). The most recent findings focused on understanding this crosstalk to explain the mechanisms leading to the involution of bone–muscle unit.

The interaction between muscle and bone

The anatomical and functional connection between the muscle and bone has fostered the idea that the interaction between these tissues depends on

mechanical forces, where muscle traction acts as a stimulus to promote the balance of bone remodeling processes. Mechanosensitivity is a complex phenomenon resulting from the combination of physical forces and stimuli that act on various compartments of bone cells, such as plasma membrane, cytoskeleton, cilia, and ion channels, thus determining a variety of biological responses (17). Recent findings indicate that the crosstalk between muscle and bone is also mediated by reciprocal paracrine and endocrine signals (Table III) (17-19).

The assessment of changes in circulating levels of factors involved in muscle-bone interaction could be a helpful tool in the diagnostic workup of osteosarcopenia. Serum myostatin, a muscle specific biomarker that suppresses muscle growth and bone formation, is significantly higher in sarcopenic than nonsarcopenic individuals (20). Circulating levels of IGF-1, a growth factor that promotes muscle growth and osteogenesis, correlate with lean body mass and are significantly decreased in osteoporotic women with hip fracture (21) and in patients with moderate sarcopenia (22). In addition, irisin, a pro-osteogenic protein released by muscle cells during physical exercise, is decreased in subjects with muscle dysfunction (23). However, it is reasonable to assume that a combination of multiple serum biomarkers could more accurately represent the osteosarcopenia status considering the complex mechanisms underlying the muscle–bone interaction.

Cell senescence

Mesenchymal stem cells can differentiate in various cell types, such as myocytes, and osteoblasts, but their intrinsic plasticity declines, their proliferation is impaired, and their differentiation into adipocytes prevails with advancing aging (16). Moreover, senescence-associated secretory phenotype may have disruptive effects on the surrounding microenvironment, thus compromising the tissue homeostasis (24). Therefore, bone formation is affected because it depends on the number and activity of osteoblasts during remodeling. Similarly, the number of satellite stem cells that are involved in the normal growth of muscle and their regeneration after injury or disease are significantly reduced. The aging process of the muscle starts with progressive denervation of the motor units leading

Table I. *Clinical definition of sarcopenia.*

Working group	Muscle mass	Muscle strength	Physical performance
EWGSOP (2010)	ASM/height^2 Men: < 6.52 kg/m^2 Women: < 5.44 kg/m^2	Grip strength Men: ≤ 30 kg Women: ≤ 20 kg	Gait speed: < 0.8 m/s
IWGS (2011)	ASM/height^2 Men: ≤ 7.23 kg/m^2 Women: ≤ 5.67 kg/m^2	---	Gait speed: < 1 m/s
AWGS (2014)	ASM/height2 Men: < 7.0 kg/m^2 Women: < 5.4 kg/m^2	Grip strength Men: < 26 kg Women: < 18 kg	Gait speed: < 0.8 m/s
FNIH (2014)	ASM/BMI Men: < 0.789 Women: <0.512	Grip strength Men: < 26 kg Women: < 16 kg	Gait speed: < 0.8 m/s
EWGSOP2 (2018)	ASM Men: < 20 kg Women: < 15 kg ASM/height^2 Men: < 7 kg/m^2 Women: < 5.5 kg/m^2	Grip strength Men: ≤ 27 kg Women: ≤ 16 kg	Gait speed: < 0.8 m/s Chair stand: >15 s for five rises SPPB: ≤8 point score TUG: ≥20 s 400 m walk test: non-completion or ≥6 min for completion

to the reduction of type II fibers (fast twitch), which are gradually replaced by type I fibers (slow twitch) and adipose tissues (16). Muscle biopsies of subjects with osteoporotic hip fracture reveal a lower number of satellite cells and a higher percentage of myostatin positive fibers, which are findings that may be considered a hallmark of sarcopenia (25).

Metabolic changes

The presence of localized fat in the muscle and bone marrow is a distinctive sign of aging, and the secretion of fatty acids and adipokines has negative effects on the surrounding tissues and affects muscle-bone crosstalk (2). High circulating levels of proinflammatory adipokines, such as IL-6, TNFα,

Table II. *Secondary causes of osteosarcopenia.*

Endocrine and metabolic diseases	Type II diabetes mellitus, early menopause, hypogonadism, thyroid disorders, hypercalciuria, hypercortisolism, Paget's disease
Inflammatory diseases	Rheumatoid arthritis
Malignant diseases	Solid tumors, neoplastic disorders of hematological origin
Organ failure	Heart, lung, liver, kidney, or brain
Medications	Glucocorticoids, chemotherapeutics, heparin, antiepileptics, aromatase inhibitors, 'gonadotrophin-releasing hormone agonists, levothyroxine
Nutritional status	Malabsorptive conditions, cachexia, excessive weight loss, low intake of protein and calcium, vitamin D deficiency, alcohol excess,
Activity and life style	Prolonged hospitalization, institutionalization, prolonged weightlessness, sedentary lifestyle, socioeconomic status smoking

Table III. *Factors involved in muscle-bone crosstalk.*

Muscle to bone	Bone to muscle
β-aminoisobutyric acid	Bone morphogenetic protein 1
Bone morphogenetic protein 1	Insulin-like growth factor 1 (IGF-1)
Brain-Derived Neurotrophic Factor	Matrix metalloproteinase-2
Decorin	Osteoactivin
Fibroblast growth factor 2 (FGF-2), FGF 21	Osteocalcin
Follistatin	Osteonectin
Insulin-like growth factor 1 (IGF-1)	Prostaglandin E2
Interleukin 6 (IL-6), IL 7, IL-15	Receptor activator of nuclear factor kappa-B ligand
Irisin	Transforming growth factor β
Leukemia inhibitory factor	Wingless-Type MMTV Integration Site Family, Member 1 and 3a (Wnt1, Wnt3a),
Matrix metalloproteinase-2	
Myostatin	
Osteoactivin	
Osteoglycin	
Osteonectin	

adiponectin, and leptin, have been found in subjects with sarcopenia and osteopenia. Palmitic acid, the fatty acid most expressed by marrow adipocytes, has lipotoxic effects on the survival and function of osteocytes, osteoblasts, and myotubes (16).

Diabetes mellitus is associated with a significant impairment of muscle tissues, with atrophy of type II fibers, insulin resistance, lipotoxicity, reduced synthesis of glycogen, and mitochondrial dysfunction. Chronic hyperglycemia, oxidative stress, and in particular "advanced glycation end products" induce apoptosis, suppress the differentiation of myogenic genes, and favor bone mass loss (16). Clinical studies concerning nutritional factors have reported that low protein intake and low levels of vitamin D are associated with a reduction in muscle mass and BMD. Moreover, the adequate calcium supply is required for the maintenance of both structural integrity of bone hydroxyapatite and signal transmission at neuromuscular level (2).

Genetics and epigenetics

Research on a shared etiology between osteoporosis and sarcopenia reported that approximately 60% to 70% of risk factors are heritable. Some genetic variants involved in the etiopathogenesis of osteosarcopenia have recently been described that concern myostatin, a negative regulator of muscle mass and bone formation; α-actinin 3, a binding protein involved in the assembly of actin in the thin filaments of the sarcomere; myocyte enhancer factor 2C, which has a role in myogenesis and in maintaining the differentiated state of muscle cells; transcription factors and other genes involved in energy metabolism of myoblasts and osteoblasts (16). MicroRNAs and epigenetic alterations related to cell senescence, for example, the loss of heterochromatin, genome instability, DNA methylation, and changes in RNA expression, have been described as important players in controlling bone and muscle interaction (16). However, the knowledge on epigenetic alterations in musculoskeletal pathophysiology derives from studies that examine these tissues separately, and the possibility of using these alterations as biomarkers is still unclear.

DISCUSSION

Disability and mortality after hip fractures are notably increased in patients affected by osteosarcopenia. Physicians should consider the health status of the muscle and bone to identify frail patients who could benefit from personalized therapeutic interventions aimed at reducing the risk of adverse outcomes. Major limitations occur when the severity of sarcopenia must be determined because the physical performance assessment is impracticable in bedridden patients. In this context, the availability of reliable biomarkers could be very useful for the diagnostic workup. The measurement of circulating levels of molecules involved in bone-muscle interaction, genetic and epigenetic factors, and metabolic alterations seem to be promising markers, but further studies are needed to establish their diagnostic accuracy.

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A ninety-minute football match increases hamstring flexibility in professional players

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Flexibility is an integral component in any conditioning program. Flexibility has been defined as the ability of a muscle to lengthen and allow one or more joints in a kinetic chain to move through a range of motion. The lack of flexibility of the hamstring muscle group has been associated with a higher risk of non-contact muscle injury, and for several other conditions, such as changes in lumbopelvic rhythm, greater thoracic kyphosis and lumbar flexion, and lower back pain. The present study explored the effects of a 90-minute soccer match on hamstring group flexibility. Our study shows that a 90-minute football match favorably impacts the flexibility of the hamstring muscle group. Flexibility is a modifiable risk factor for muscle strain injury. It remains to be ascertained how long this effect lasts, and whether it may be associated with the risk of developing or avoiding noncontact injury to the hamstring muscle group.

Flexibility is an integral component in any conditioning program. Flexibility has been defined as the ability of a muscle to lengthen and allow one or more joints in a kinetic chain to move through a range of motion (1). Some of the proposed benefits of enhanced flexibility are reduced risk of injury, pain relief, and improved athletic performance (2).

Non-contact hamstring muscle injury are caused by a multitude of factors (3). These include a lack of hamstring strength (4) and flexibility (5), though these findings are not univocal (6). Poor flexibility is nevertheless considered a modifiable risk factor for muscle strain injury (7).

The lack of flexibility of the hamstring muscle group has been associated with a higher risk of non contact muscle injury (8), and for several other conditions, such as changes in lumbopelvic rhythm (9), greater thoracic kyphosis and lumbar flexion, and lower back pain (10). For these reasons, hamstring muscle flexibility is routinely tested in athletes (11). The present study explored the effects of a 90 min soccer match on hamstring group flexibility.

MATERIAL AND METHODS

Ethics approval for the study was granted by the University of Athens Physical education department. Written informed consent was obtained from each player or their legal guardian prior to data collection. Participants were blinded to the study hypothesis. The study was carried out in accordance with the Declaration of Helsinki.

Twenty-two professional football players (age: 19.3±2.9; height 185.0±8.7 cm; body mass 81.6±6.7 kg) participated in this study. All players were right

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leg dominant (defined as their preferred 'kicking' leg). Subjects were included in the study if they were not injured or rehabilitating from an injury at the time of testing.

The game was played in the second half of the competition season. Goniometry was used to assess posterior thigh flexibility with the Active Knee Extension (AKE) test. All the athletes were always measured by the same physiotherapist who has several years' experience, including experience in measuring posterior thigh flexibility using the AKE test. In addition, the intra-rater reliability was tested. The athletes were asked to inform the sports physical therapist of any hamstring-related complaints they may have had. No changes or adjustments were made to the training schedule of the athletes or their nutrition habits.

Each athlete was positioned supine with both knee and hip flexed to 90° (the 90/90 position) on an examination couch. The opposite leg was placed flat on the couch with the knee fully extended and maintained in this position throughout the test. The lateral epicondyle of the femur was palpated, and the goniometer was centered over it. The lateral malleolus of the tibia and the greater trochanter of the femur were then marked. The arms of the goniometer were aligned with the proximal and distal landmarks.

The athlete was instructed to actively extend the knee being tested until this was possible, with the hip was kept at 90° of flexion. The stationary arm of an inclinometer [a double-arm 30-cm clear plastic inclinometer Lafayette (Instrument Company, Lafayette, Indiana)] was aligned along the femur with reference point at the greater trochanter of the femur; the axis of movement was placed at the lateral femoral condyle at the knee joint and the moving arm was aligned with the lateral malleolus. Angles were measured to the nearest degree, on both sides, representing the active range of motion (AROM) measurement [90/90 position = 0 degrees (inflexible); full knee extension = 90° (flexible)]. Room temperature was 20°C, and athletes did not undertake any warmup before the AROM measurement.

Statistical Analysis

The Shapiro-Wilk test confirmed that the data followed the normal distribution (Table I). We tested whether flexibility in the hamstring muscle group in each player changed before and after the match, using a paired sample t-test and one sample t–test. Cohen's d index was used to estimate the intensity of the difference whenever that was necessary.

The bootstrap method (1000 bootstrap samples) was used to estimate more accurately the confidence intervals of the difference before and after the match in the variables of interest. The statistical analysis was conducted by using the statistical software SPSS 23.0.

RESULTS

Table 1 reports the average changes in hamstring flexibility before and after the football match.

There was a significant difference in flexibility of the hamstring muscle group before and after the football match (17.55±4.04 vs 20.32±4.02; t (21) = 8.440, p =.000, Cohen's d = .70). Also, the bootstrap 95% confidence interval was (-3.41, -2.18), showing an improvement of 12.42% to 19.43% from the initial flexibility (M=17.55) of the athletes. (Fig. 1).

Table I. *Difference in hamstring flexibility before and after the football match.*

	M	SD	Lower	Upper
Before the football match	17.55	4.04	-3.41	-2.18
After the football match	20.32	4.02		

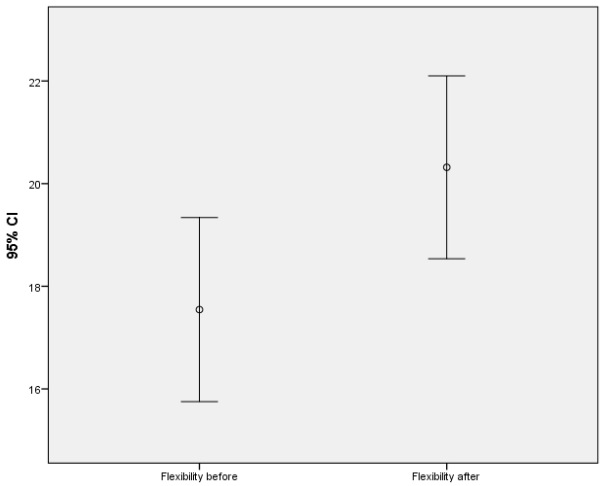

Fig. 1. *Flexibility before and after the exercise, 95% confidence intervals.*

DISCUSSION

The present study shows that a 90-minute football match favorably impacts the flexibility of the hamstring muscle group. Flexibility is a modifiable risk factor for muscle strain injury (12, 13). A recent study provided theoretical support for this suggestion considering the effects of hamstring flexibility on isometric knee flexion angle-torque relationship. That study demonstrated that subjects with poor hamstring flexibility exhibited a greater knee flexion angle for the maximum knee flexion torque in an isometric contraction test compared to subjects with normal hamstring flexibility. This would indicate that an athlete with poor hamstring flexibility may have shorter optimum hamstring muscle lengths compared to athletes with normal hamstring flexibility. Shorter optimum muscle length may result in higher muscle strain for the same range of motion, and thus increase the risk for hamstring strain injury (14).

Clinical studies on the effect of hamstring flexibility on the risk for hamstring muscle strain injury paint an inconsistent picture. A case-control study tested hamstring flexibility and concentric and eccentric strength at 60°/s and 180°/s in 16 athletes who had hamstring strain injuries within the past 18 months and 16 sports and dominant leg matched controls without injury (7). There was a significant difference in hamstring flexibility between injured and matched control groups. English soccer players who sustained a hamstring muscle injury exhibited significantly less hamstring flexibility measured before their injuries compared to their uninjured counterpart: poor hamstring flexibility would be a risk factor for hamstring muscle strain injury (15).

Several other studies showed no significant difference in hamstring flexibility prior to hamstring muscle strain injuries between injured and uninjured athletes. Elite Australian football players who had recurrences of hamstring muscle strain injury appeared to have better hamstring flexibility in comparison to their counterpart without recurrence of the injury (16). The inconsistency among these studies may result from differences in control group, control of other risk factors, and injury risk measures in study designs (17). Further studies with improved research designs are needed to determine the effects of flexibility on the risk of hamstring muscle strain injury.

Several authors have investigated the relationship between hamstring flexibility and hamstring injury (14, 18). Worrell et al. (7) and Liemohn (14) reported that hamstring-injured subjects were less flexible than noninjured subjects. In contrast, Burkett (19) reported no difference in hamstring flexibility between hamstring-injured and noninjured subjects. In addition, flexibility has been assessed in several ways. Ekstrand and Gillquist (20) reported no evidence of a statistically significant relationship between hamstring flexibility and hamstring injury. Burkett (19) used the Wells sit-and-reach method. Liemohn (14) and Ekstrand and Gillquist (20) used the straight-leg-raise method. Worrell et al. (7) used the passive-knee-extension test.

Only two research groups reported reliability data to support their method of assessing hamstring muscle length (3). Worrell et al. (7) reported the use of a passive-knee extension test (N = 20, test-retest Pearson product moment coefficient of r = 0.98). During the passive knee extension test, each subject is placed supine with the hip positioned at 90° of flexion. The hip is then stabilized in this position by the subject placing both hands around the distal thigh just proximal to the knee joint with the fingers interlocked while maintaining the foot in plantar

flexion. The opposite leg is maintained at 0° of hip flexion. A universal goniometer is used to determine the hip position. To determine hamstring flexibility, the knee is passively extended by the tester while the hip is maintained at 90° of flexion by the subject. The stationary arm of the goniometer is then placed parallel to the midline of the femur and the movable arm is placed parallel to the midline of the fibula. The point in the knee range of motion where resistance is encountered while maintaining the hip at 90° is determined as the end of hamstring range of motion. This evaluation technique is similar to the active-knee-extension method recommended by Gajdosik and Lusin (21) to determine hamstring flexibility.

Ekstrand et al. (20) reported the use of the straight-leg raise (SLR) method to assess hamstring muscle length (N = 22, coefficient of variation = 1.9±.07%). The SLR test for hamstring flexibility assessment may be confounded by pelvic rotation (7) and foot position (22). The Wells sit-and-reach test for hamstring flexibility assessment may be confounded by the flexibility of the upper extremity and lumbar and thoracic spine.

Ekstrand and Gillquist (20) reported that 180 soccer players had greater hamstring flexibility (SLR) than a group of 86 non-players, with no association between past injury and muscle tightness. In contrast, Worrell et al. (7) reported that the hamstring-injured group's injured limb was significantly less flexible than the noninjured limb ($p < 0.05$). Also, the authors reported that both hamstring muscles of the injured group were less flexible than the hamstring muscles of the noninjured group ($p < 0.05$). It is plausible that a less flexible extremity was present prior to hamstring injury. Inflammation and adhesion occur following muscle injury (22). Furthermore, calcification within the hamstring muscles following muscle strain has been documented (16). Therefore, loss of hamstring flexibility is a possible sequela of a hamstring muscle injury. Hence, greater passive flexibility may allow muscles to effectively resist potentially injurious lengthening forces. This appears to be the case for exercise-induced muscle damage (17, 23). There is indirect evidence that a rightward shift in the angle-torque relationship for the hamstrings (greater strength at longer muscle lengths) protects against muscle strain injury.

Clinical implications

The main finding of our study was that fatigue induced by a 90-minute football match does not negatively affect the flexibility of the hamstring muscle group in well trained footballers. Data from the present study support the finding that improvements in hamstring flexibility take place after a 90 min football game.

Limitations

The present investigation presents several limitations. First, to determine the specific level of an uncomfortable sensation in the hamstring muscle during the passive tests, a visual analog pain scale could be used. Second, to determine the involvement of several muscles in the tests used, electromyography could be used. Third, the mechanical properties of the hamstring muscles could be assessed as acute fatigue of the hip flexor muscles could result in greater hip ROM in the passive tests.

The relationship between passive hamstring flexibility and risk of hamstring strain has not been specifically examined in a large sample of athletes from a sport with a high incidence of hamstring strains (e.g. Australian rules football or soccer). While hamstring flexibility may not be a risk factor for subsequent strain injury in Australian rules football players (24), the sample was small (n=37), and the sit and reach test used to assess hamstring flexibility is regarded as a less specific measure of hamstring flexibility than the SLR test.

The literature reports controversial findings regarding the association between hamstring flexibility and the risk of injury. Many prospective studies demonstrated no relationship between flexibility of the knee flexors and hamstring injury (25). On the other hand, studies involving professional European football players showed an association between flexibility values obtained in preseason training and the injuries suffered during the season (15).

Our study showed that a 90-minute football match can positively influence the flexibility of the hamstrings muscle group in professional football players. It remains to be ascertained how long this

effect lasts, and whether it may be associated with the risk of developing or avoiding noncontact injury to the hamstring muscle group.

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The metabolic effects of quinolones and steroids on human ligament cells: an *in vitro* study

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The toxic effects of fluoroquinolones and steroid on tendon cells have been well established, but their role on human ligamentocytes remain unclear. We have investigated the effects of ciprofloxacin and methylprednisolone on human anterior cruciate ligamentocytes after 7 and 14 days of culture. We evaluated cell viability, Annexin V-FITC/PI assay, senescence-associated β-galactosidase staining, and collagen type I detection. Regarding quinolones administration, we observed that ligament cells treated with ciprofloxacin have characterized by a significantly decrease of cell viability and collagen type I expression and an increase of apoptotic cells. In cells treated with high dose of steroid we observed a significantly decrease of cell viability and collagen type I expression and the presence of senescent cells. Therefore, ciprofloxacin and methylprednisolone might have cytotoxic effects on ligamentocytes by two distinct mechanisms. Quinolones seem to induce cell apoptosis, while steroids might be able to induce cellular senescence. Hence their use should be avoided in athletes and in orthopedic surgery.

Fluoroquinolones are used in the treatment of a wide range of infections due to their tissue penetration and their high volume of distribution (1). Moreover, the use of fluoroquinolones as prophylaxis and therapy in orthopedic surgery has increased considerably in the last years cause the development of beta-lactam resistance emerged from the latest literature (2). However, the toxic effects of fluoroquinolones on tendon cells in vitro have been well established (3). Previous studies have demonstrated that apoptosis of tenocytes is involved in the pathogenesis of tendon destruction. It has been well established that levofloxacin and ciprofloxacin through a time and concentration dependent mechanism are able to increase the apoptosis marker caspase-3 in cultured human tenocytes (4). For these reasons, programmed cell death must be considered a crucial event in the pathogenesis of fluoroquinolone induced tendinopathies. Ligaments and tendons have similarities in structure, components, characteristics and function.

Indeed, they are characterized by low vascularization, similar matrix components and transmembrane and intracellular signaling proteins (5). Therefore, based on their structure, fluoroquinolones may also have cytotoxic effects on ligamentocytes as those demonstrated on tenocytes. Rabbit Anterior cruciate ligament (ACL) cells were treated with levofloxacin at different concentrations. The authors found that levofloxacin enhanced dose-dependent ACL cell apoptosis and decreased extracellular matrix, suggesting a potential adverse effect of fluoroquinolones on ligaments (6). To date, corticosteroids are widely administered to treat various diseases.

Local and/or systemic corticosteroids have been frequently used to treat acute or chronic joint effusion after trauma, osteoarthritis or during

Key words: fluoroquinolone, glucocorticoid, ligament, apoptosis, cell metabolism, senescence

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anesthesia and analgesia procedures for primary ACL reconstruction (7, 8). Studies revealed degenerative changes, reduced synthesis of type I collagen and proteoglycans, as well as decreased tenocytes proliferation, viability and metabolism following glucocorticoid exposure.

Hence, adverse events of corticosteroid treatments, as impaired tendon healing and rupture, have been reported (9, 10). Despite their extensive use in surgical and non-surgical treatment by orthopaedic and sport trauma physicians, the effects of fluoroquinolones and corticosteroids on human ACL *in vitro* or *in vivo* are still unknown. Therefore, our study aimed to demonstrate the effects of fluoroquinolones and corticosteroid on human ACL cells in culture.

MATERIALS AND METHODS

Patients were treated at the University Hospital "Ospedali Riuniti" of Ancona (Italy). Before the beginning of any study-related activities, each patient signed informed consent. The study was approved by our institutional review board.

Isolation and culture of ACL cells

Ligamentocytes were isolated from the ACL of 10 human donors (age 64.5±5.7), 7 males and 3 females, during knee arthroplasty surgery. During surgery, waste fragments of ligament that were not of any medical use, were collected and immediately transferred to Laboratory of the Clinical Orthopaedics, Università Politecnica delle Marche (Italy) for histological analysis.

The ACLs were removed from knee joints under aseptic conditions, diced into pieces approximately 1 mm in diameter and then were cultured in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (all reagents from GIBCO® Life Technologies Corporation, USA) at 37°C were incubated at 37°C in a humidified, CO_2-controlled (5%) incubator. Medium was changed twice a week. After cells reached 80% of confluency, they were collected by treatment with 0.25% trypsin EDTA (Sigma-Aldrich, Milan, Italy) and subcultured at 1:3 dilutions under the same culture condition. Cells were used at the 3rd passage.

It is mandatory to consider that the plasma concentration of ciprofloxacin after a single oral administration and after intravenous injection is respectively around 2 μg/ml and 6 μg/ml. However, it has been reported an accumulation of ciprofloxacin in tissue up to 20-fold the plasma concentration (11). For these reasons, with the purpose to reflect in vivo conditions, we decided to use 60 μg/ml of ciprofloxacin. On the other hand, it has been reported that 1μM of steroids reflects plasma concentration of methylprednisolone after a single oral administration. However, higher concentrations as 100 or 400 μM might be potentially reached with intra-articular injection (12). Therefore, we decided to use 1, 100 and 400 μM of methylprednisolone.

We constituted four groups of cells, two groups were cultured with ciprofloxacin, two with methylprednisolone as described by Fig. 1. The medium has been changed every three days. In group

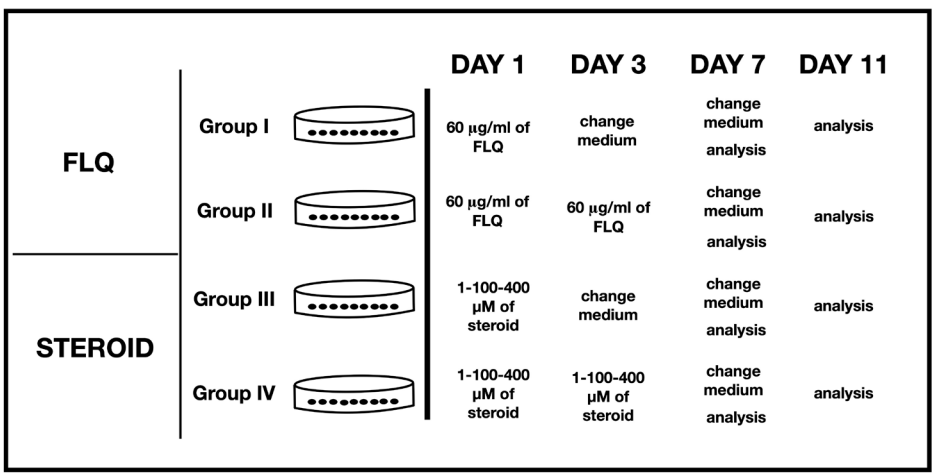

Fig. 1. *Design of the study. FLQ: fluoroquinolones.*

II and group IV, it has been added a second dose of drug at day 3.

Cytotoxicity measurement

Cell survival was determined by MTT assay. Culture medium was replaced with a 10% sterile filtered solution of MTT 5 mg/ml in DMEM without phenol red. After an incubation at 37◦C for 4 h, the MTT solution was discarded and the dark blue formazan crystals accumulated in the cells were dissolved by adding 1 ml of solvent (0.01N HCl in absolute isopropanol), and quantified spectrophotometrically (BioPhotometer Plus, Eppendorf, Hamburg Germany) at 550 nm. The results are reported as a percentage of live cells compared to control cultures.

Collagen type I detection

Collagen type I in ligament cells were quantitatively determined by a monoclonal antibody. The cells were fixed with 4% paraformaldehyde solution and then incubated with the monoclonal antibody anti- type I collagen 5 µl/ml (Imnunological Sciences, Rome, Italy). The reaction was measured with Dako EnVision + Dual link System-HRP kit (Agilent, Santa Clara, CA, USA) and was visualised using a LEICA DMIL (Leica Microsystem, Wetzlar, Germany) optical microscope. The number of

Table I. *MTT test and type I collagen detection.*

			MTT (% of viable cells) (mean ± SD)			Type I collagen (% of expression) (mean ± SD)		
			7 day	**14 day**	**p value**	**7 day**	**14 day**	**p value**
FLQ	Group I		62±5	63±4	0.57	20 ±4	22±7	0.44
	Group II		44±4	48±13	0.41	5±3	6±2	0.52
	p value		0.01	0.03		0.01	0.02	
Steroid	1 µM	Group III	95±8	89±12	0.39	46±5	67±6	0.02
		Group IV	93±7	87±17	0.32	34±2	57±4	0.02
		p value	0.42	0.51		0.03	0.04	
	100 µM	Group III	83±12	78±6	0.41	29±4	32±6	0.38
		Group IV	80 ±8	72±6	0.29	19±3	21±5	0.35
		p value	0.44	0.27		0.02	0.03	
	400 µM	Group III	54±1	26±8	0.02	4±1	2±1	0.02
		Group IV	54±6	20±5	0.01	3±2	1±0.5	0.04
		p value	0.54	0.34		0.04	0.03	

FLQ: fluoroquinolone; SD: standard deviation.

collagen type I-positive cells and the total number of cells were counted in 5 fields of view (10X) per sample.

Annexin V-FITC/PI assay

Early apoptotic, late apoptotic and/or necrotic cells was quantified using the annexin V-FITC and propidium iodide method (TACS Annexin V FITC Kit, Trevigen, Gaithersburg, MD, USA). Briefly, cells were incubated for 20 min at room temperature with 2 µl annexin V-FITC, supplemented with 10 µl of PI, and finally analysed by flow cytometry (FACSCalibur Becton Dickinson, CA, USA), using FL1 channel for annexin V-FITC binding and FL3 channel for PI staining.

Senescence-associated β-galactosidase staining

Senescence-associated hypertrophy leads to increased lysosomal number and activity. It is a feature which is exploited in one of the most widely used assays to identify senescence: the activity of lysosomal β-galactosidase (SA-β-gal). SA-b-gal-positive cells were detected according to a manufacturer protocol and were visualised using a LEICA DMIL (Leica Microsystem, Wetzlar, Germany) optical microscope. The number of β-galactosidase-positive cells and the total number of cells were counted in 5 fields of view (10X) per sample.

Statistical analysis

For quantitative data, each assay was repeated at least three times independently. The results were expressed as mean ± standard deviation (S.D.). Different groups were compared by using ANOVA. $P<0.05$ was statistically significant.

RESULTS

MTT test and type I collagen expression

The MTT test and type I collagen expression were reported in Table I. It is worth mentioning that ligament cells treated twice with ciprofloxacin were characterized by a significantly decreased of cell viability respect to group I. On the other hand, ligamentocytes would seem to not be affect by the number of administrations of steroid. Indeed, it has not been reported statistically significant differences in term of cell viability at 7 and 14 days between group III and group IV.

However, at the highest concentrations (400 µM) was observed at a significantly decreasing cell vitality between day 7 and day 14 as showed in Fig. 2. In both groups of ciprofloxacin, we observed an increase of type I collagen expression at 14 days respect those at 7 days, even though type I collagen expression was significantly lower with double dose of antibiotic. In group III and IV type I collagen expression depends on the dose of steroid and the number of doses. It is interesting to note that with the highest dose of steroid at 14 day we observed a decrease of type I collagen expression, while at 1µM of methylprednisolone was observed a statistically

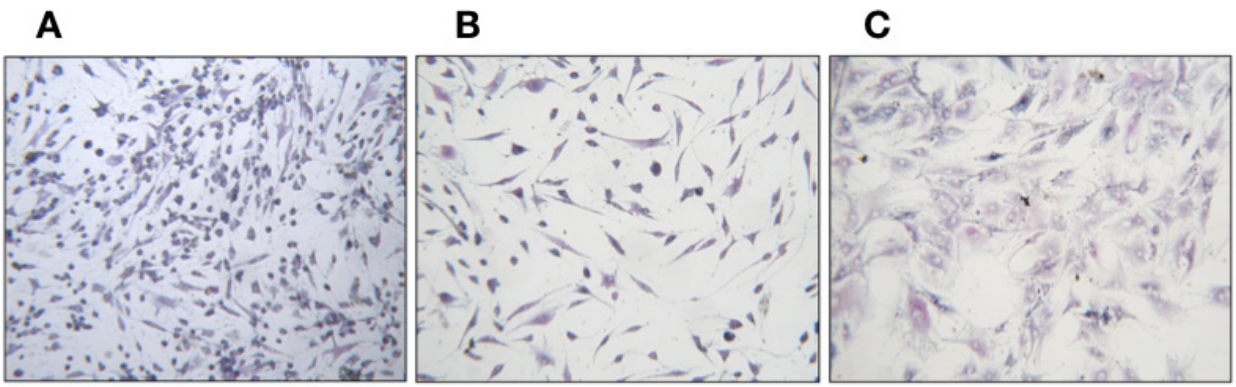

Fig. 2. *Mitochondrial dehydrogenase activity (MTT assay) optical microscope images (10X) of ligament cells after a 7-day of incubation.* ***A****): Ligament cells without ciprofloxacin treatment;* ***B****): Ligament cells of group II treated with 60 µg/ml ciprofloxacin reveal a decrease of cells number;* ***(C****): Ligament cells of Group IV treated with 400 µM of methylprednisolone reveal a decrease of cells number and lost their fibroblast-like shape.*

significant increase of the content of collagen type I between day 7 and day 14.

Annexin V and propidium iodide (PI)

Regarding wells treated by ciprofloxacin, we observed an increase expression of annexin V at 7 days in group I and II respectively of 2.19±0.5% and 4.75±1% compared to control wells (Fig. 3). At 14 days, no statistical differences have been observed between control and fluoroquinolone groups ($p>0.05$). No differences in terms of PI expression have been observed in both groups compared to controls at 7 and 14 days ($p>0.05$). Regarding steroid groups we did not observe significance differences in both groups compared to controls in terms of Annexin V and PI expressions in relation of the dose used and time of analysis ($p>0.05$).

β-galactosidase assay

SA-β-gal-positive cells were not observed in group I and II at 7 and 14 day of culture. In group III and IV we did not report SA-β-gal-positive cells in wells tested to 10 and 100 μM of methylprednisolone. Distinct effects were found, with the highest concentration levels. Indeed, at day 7 the percentages of SA- β-gal-positive cells were respectively 4±1% and 12±3% in cells treated once and twice with 400 μM of steroid. At 14 days, these percentages raised respectively to 10±2% and 33±4%.

DISCUSSION

Tendon tissue alterations due to fluoroquinolones and steroids have been investigated by many studies conducted in vitro (13, 14, 15, 16). This is the first study to demonstrate that fluoroquinolone antibiotic ciprofloxacin and steroid have a direct effect on human ligament cells metabolism. The maintenance of normal ligament function depends on a balance between catabolic and anabolic fibroblast metabolism. This balance between cell anabolism (ground substance synthesis, cell proliferation) and cell catabolism (cytokine synthesis, protease activity) is necessary for ligament matrix turnover. Ligament fibroblasts responds to microscopic and macroscopic injury by cell proliferation, migration, and synthesis of matrix ground substance. Thus, in normal and pathologic conditions, ligament function is directly dependent on fibroblast metabolism.

The decrease matrix synthesis, combined with the concomitant decreased cell proliferation and increased of apoptosis observed in our experiments, suggests a mechanism by which ciprofloxacin might predispose ligament to injury. It has been well established that quinolones exert their antibacterial effect by preventing bacterial DNA from unwinding and duplicating through the inhibition of the activity of the type II topoisomerases, gyrase and topoisomerase IV. Ciprofloxacin has also been reported to interfere with mitochondrial DNA transcription leading to oxidative stress and impaired cell replication (17).

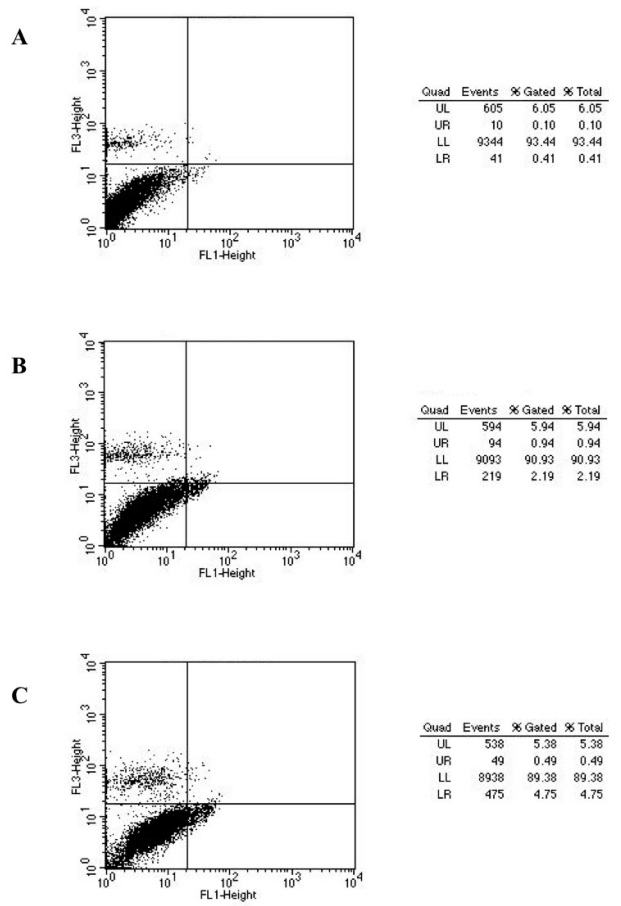

Fig. 3. *Dot plot of annexin V-FITC/PI assay obtained from ligament cells after a culture period of 7 days; **A**): control cells; **B**): ligament cells treated once and; **C**): twice with 60 μg/ml of ciprofloxacin. Apoptotic cells are located in the lower right square.*

According to the published literature and considering our results, we believed that ciprofloxacin through its inhibition of mitochondrial DNA transcription in ligament fibroblast might be able to alter cellular metabolism making it more susceptible to ruptures. We believe that quinolones might not be cytotoxic for the ligamentocytes evidenced based on the fact that, propidium iodide was comparable to checkups. At the same time, ciprofloxacin can reduce cellular anabolic metabolism. Hence, it has been reported a reduced synthesis of type I collagen and a reduced number of ligament cells probably determined by an increase of apoptosis. Interestingly, SA-β-gal-positive cells were not observed in both group of quinolones. For these reasons we believe that ciprofloxacin might not be able to make ligament cells senescent. Indeed, when the administration of quinolone stopped, the cells were able to resume their metabolism as the synthesis and secretion of type I collagen.

Based on our results, the effect of steroid on ligament cells metabolism is different from that observed with quinolone. We clearly reported that steroid might not be able to induce apoptosis or necrosis of ligament fibroblast. Moreover, the values of MTT test show that cell viability is reduced, especially with the highest dose of steroid. Together, taking these results, we hypothesized that steroids might be able to induce alterations of mitochondrial and cell metabolism reducing the activity of mitochondrial reductase. In addition, the oxidative stress enhanced by respiratory chain dysfunction might be able to reduce cell proliferation and the synthesis and secretion of type I collagen. In particular, the production of type I collagen is impaired with highest dose of steroid and in case of double administration.

Interestingly, ligament cells exposed to high dose of steroid, show SA-β-gal-positive cells especially in case of double administration. Poulsen et al. have shown that corticosteroids induce senescence in tenocytes with the activation of the p53/p21 pathway. Senescent cells produce abnormal extracellular matrix, with growth factors, cytokines and matrix degradation enzymes implicated in the pathogenesis of tendinopathies (16). Considering our results, differently from quinolones, steroids might be able to induce cellular senescence. Indeed, while the production of type I collagen seems to resume with a low dose of steroids after 14 days of culture, in both groups with high dose of steroids we reported a deficit of type I production that might reflect the presence of senescent ligament cells no longer able to produce collagen.

Further studies, such as the analysis of p53/p21 and p38/p16 pathways, are necessary to determine the pathway of cell metabolic damage following administration of methylprednisolone. In addition, the effects of methylprednisolone and ciprofloxacin treatments on ligament tissues should be investigated in animal models. Finally, efforts should also be made to prevent the deleterious effects of methylprednisolone and ciprofloxacin treatments by adding simultaneous application of other treatments.

Although this is an **in vitro** study, we suggest avoiding quinolones in athletes, especially in sports with many directional changes such as football and skiing. Their use should be limited to the strict minimum necessary in patients with ligamentous lesions treated conservatively or surgically and in pre-operative antibiotic prophylaxis of orthopedic surgery on ligaments. Regarding the use of steroid, we suggest to limiting the use of intra-articular corticosteroid injection in athletes and patients with ligaments treated conservatively or surgically.

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Induced membrane by silver-coated knee megaprosthesis: keep or toss?

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In the orthopaedic field the foreign body reaction is well known for therapeutic purposes in the alleged Masquelet technique consisting of segmental bone loss two-stage reconstruction. The induced membrane creates advantageous local conditions that promote bone graft remodeling and osteointegration. The aim of our study was to describe the first two cases in Literature of induced membrane observed following silver-coated knee megaprosthesis reconstruction. In addition, it was our interest to evaluate their histological features.

The development of prosthesis for wide resection offered to the orthopedic oncological and degenerative surgery further therapeutic opportunities (1). Modular megaprosthesis are currently the most common extremities long bones method of reconstruction thanks to their availability, the relative ease use, the immediate fixation, the early weight bearing, the relatively rapid function recovery and the excellent cosmetic appearance (2). However they represent a complex and not without risks surgery thus solely reserved to selected cases (3). Due to improve the joint replacement procedures, the construct must be long-lasting and biomechanically steady (4). Despite the innovative materials and implant designs, these systems are affected by a high rate of complications among which infections are definitely the most serious (1); revision surgery is thus relatively frequent. Afterwards the joint replacement surgery, the homeostatic balance among the bone and the surrounding soft tissues with the current biomaterials shall be achieved. As well as all medical devices, orthopedic implants may have limited life expectancy due to the host foreign body reaction that may occur when the latter conditions are not met (5). Studies have shown that various biomechanical factors could lead whether a fibrous encapsulation or a bony covering which marks the foreign materials (6). In orthopaedics, the foreign body reaction is well known for therapeutic purposes in the alleged Masquelet technique for segmental bone loss two-stage reconstruction (7). This latter procedure is grounded on the development of an induced membrane around a polymethylmethacrylate (PMMA) spacer which is removed 6–8 weeks after placement. The membrane envelops the cavity thus created, which will then be filled with an autologous corticocancellous bone graft, or similar, in order to achieve bone reconstruction (8).

Animal studies were capable of characterizing the membrane architecture demonstrating the well-structured layered organization (9). Although in Literature there is no univocal evidence on the induced membrane role and benefits so far, previous studies have suggested that this specialized tissue creates advantageous local conditions that promote the bone graft remodeling and osteointegration (10).

Key words: induced membrane, Masquelet, megaprosthesis, knee, silver coated

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The aim of our study was to describe the first two cases in Literature of induced membrane observed following knee megaprosthesis reconstruction and to evaluate the benefits in preserving it onsite.

Case #1

A 75-years-old man with an anterior middle third Adamantinoma of the right tibia underwent wide margins tibial resection succeeded by modular Mutars megaprosthesis replacement. One year on, for the appearance of acute pain, swelling and a secreting fistula in the surgery wound middle third, the patient received revision surgery with custom-made cement spacer placement. During this last procedure, the surgeons noticed the presence of a thick membrane sent for histological examinations (Fig. 1). In addition, microbiological deep swabs that tested then positive for Staphylococcus lugdunensis and Corynebacterium jeikeium have been performed. The patient started and completed the proper antibiotic therapy. Afterwards he removed the spacer and underwent tibial megaprosthesis placement including knee and ankle articulation.

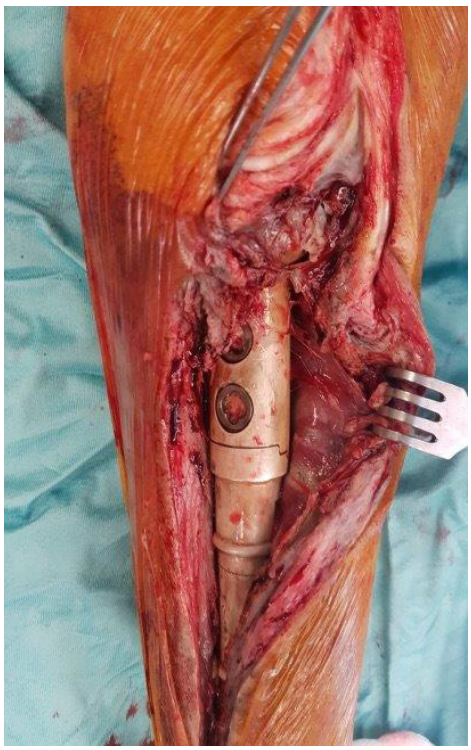

Fig. 1. *Intraoperative vision of patient #1. It is possible to notice near the retractor the presence of a hard membrane surrounding the prosthesis.*

Patient #1

Surgical specimens on histopathological membrane examination showed fibrous tissue with abundant diffuse chronic inflammatory infiltrate mostly formed by macrophages and lymphocytes (CD45+/CD68+) and granulation tissue highly vascularized. On the surface, where fibrinous exudate was focally seen, a thin and mostly attenuated layer of synovial-like cells was present. No granulomatous features were evident (Fig. 2).

Case #2

A 38-years-old woman with a high degree soft tissue sarcoma of the right leg underwent extra articular knee resection and knee arthrodesis followed by adjuvant chemotherapy. Approximately three years later the patient underwent revision surgery placing a new custom-made arthrodesis with a 20° of bending. During surgery has been noticed the presence of a membrane sent for histological examinations.

Patient #2

The histopathological analysis revealed a dense sclerotic fibrous tissue with a poor chronic inflammatory infiltrate characterized from fine, granular, black pigment deposit. The CD68+ cells were mainly observed in proximity of the surface where a synovial-like cells attenuated lining was also present. Scattered blood vessels were evident throughout the tissue. No granulomatous features were seen (Fig. 3).

DISCUSSION

The Masquelet technique consisting in induced membranes development is a surgical procedure well-known in the orthopedic field. This last technique was originally used in oncological resections although today has been replaced by megaprosthesis or custom-made prostheses usage. Currently the Masquelet induced membranes play a fundamental role in osteomyelitis and bone-loss management (11, 12).

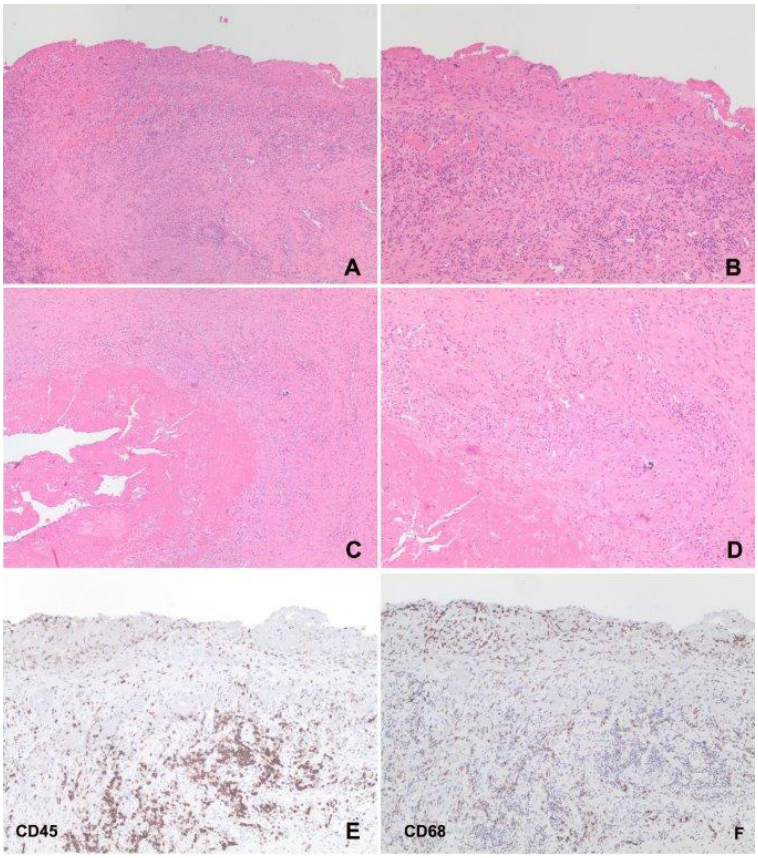

Fig. 2. *Histopathological features of biopsy specimens of patient #1. The tissue showed dense, diffuse inflammatory infiltrates (**A-D**): composed of CD45/CD68+ cells (**E** and **F**, respectively) but without granulomatous features. A dense fibrinous exudate was focally present (**C-D**).*

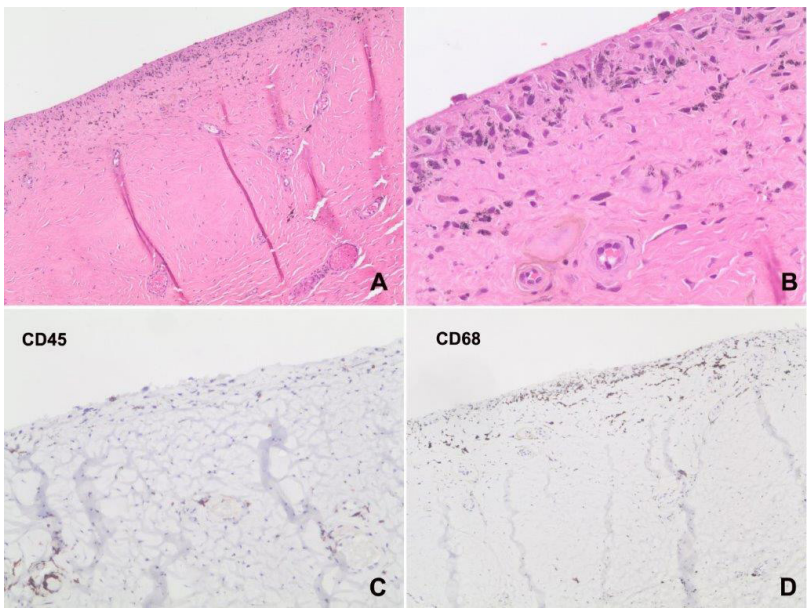

Fig. 3. *Histopathological features of biopsy specimens of patient #2 The specimens were composed of sclerotic fibrous tissue with scant chronic inflammatory infiltrates; (**A-B**): characterized by deposition of fine, granular black pigment; (**B**): with attenuates synovial-like membrane. Rare CD45+ cells were present in the tissue; (**C**): while scant CD68+ cell infiltrates were superficially seen (**D**).*

Table I. *Comparison of Masquelet induced membrane and our cases*

	Masquelet	Our cases
Sinovial like epitelium in inner layer	+	+
Collagen and fibrous tissue in middle layer	+	+
Vessel in deeper layer	+	+
Cells oriented parallel to foreign body	+	+
CD 68 + in inner layer	+	+
CD 45 +	-	+
Pigment	-	+

The megaprosthesis choice after tumor resection, complex trauma or failed arthroplasty in hip and knee surgery increased in the last decades (13, 14). The use of megaprostheses is burdened by a high complication rate, Gosheger et al reported an incidence of 12% of deep infection, 8% aseptic loosening and 1.6% stem fracture in knee replacement. The high infections incidence involving primarily the knee replacement is mostly ascribed to the poor soft tissue coverage.

Trevira Tube© (Implancast; GmbH, Buxtehude, Germany) is a polyethylene terephthalate (PET) tube over megaprosthesis that helps the muscles reinsertion. This device did not correlate to a higher risk of infections (15). In our experience we always use the Trevira Tube © in proximal femur resections and cementless megaprosthesis (16).

The silver-coated megaprosthesis choice in this sort of surgery therefore increased recently in response to the high risk of infection. This last megaprosthesis type has been demonstrated to be a safe implant that reduces the post-operative infection risk (3, 17-19). Despite this, in Literature exist conflicting reports about local and systemic silver-related complications according to different authors. Glehr et al. for instance founded a 23% of argyria incidence developed on surgical site skin and no case of systemic toxicity (20). Donati et al. didn't found any case of argyria or toxicity by silver ions in silver-coated megaprosthesis (18). Neither of the two studies founded necrosis or tissue damages due to silver absorption.

Currently there is no study in Literature that focuses on possible local reaction to the silver-coated megaprosthesis and specifically on the potential peri-implant tissue that could form. The Masquelet induced membrane has a peculiar histological layering described first by Pellisier et al. (21): the inner part is a synovial-like epithelium, the outer part is fibroblast and collagen made, finally the deeper present numerous vessels.

The histological analysis of patient #2 showed a similar macro and cito-architecture to the one described by Pellisier et al; in addition, there were the presence of black pigment probably due to silver degeneration, but in absence of necrosis. The patient #1 histopathological examination suggests a similar design by the addition of inflammatory cells likely due to the local infection. According to Henrich et al. research (22), in both our patients samples the cells located in the membrane layers were oriented parallel to the megaprosthesis similarly to the Masquelet induced membrane (Table I).

Tetsworth et al. demonstrated in the cross-section a very distinct layer of CD68-positive cells immediately adjacent to the spacer (23)one that often requires extreme solutions. This study examines a new treatment strategy for segmental bone loss using patient-specific 3D printed titanium cages in conjunction with the Masquelet technique.\n Methods: \n. The study was composed of a clinical observational case series, and a basic

science investigation to evaluate the biological activity of the induced membranes using histology, immunohistochemistry (IHC. In our samples the CD68+ cells were in the inner layer of the membrane.

The expression of CD68+ is characteristic of the monocyte lineage and is central in playing a role in the foreign body reaction, as shown by Klinge et al. (24). Tetsworth et al. also demonstrated the possible association of these macrophages with osteogenesis and therefore the bone formation. Whether these data should be verified, the megaprosthesis induced membranes, if spared, could favor the bone integration of the megaprosthesis and consequently decrease the risk of aseptic loosening and stem fractures.

Gindraux et al. (25) studied the cytometric composition of cell cultures derived from the induced membrane describing the CD expression: CD73, CD90 and CD105 were represented, in contrast with the CD45. In our samples we did not perform cytometric analysis, but there was a positivity to CD45 antibody mostly located near the vessels indicative of chemotaxis. The membrane isolates the prosthesis from the surrounding soft tissues and thus might play a protective role against future infections. Further studies will have to investigate these aspects for greater certainty.

Our histological analysis suggested that the silver-coated megaprosthesis could produce an induced membrane exempt from any sign of necrosis or tissue damage. To the authors knowledge this is the first study that offers this unique connection. An additional comparative point in between our induced-membrane and the Masquelets ones could have been the search for growth factors; unfortunately, in our study we did not collect articular fluid or synovial samples for further research.

If future studies should confirm this parity, we believe that surgeons could preserve this membrane during knee megaprosthesis revision surgery and use it as a bioreactor chamber to promote osteointegration and stability to reduce any mechanical complications.

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Biomechanical study of shaft fracture with the butterfly fragment: software simulation

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The specific traumatic mechanism that leads to the formation of the butterfly fragment is debated in literature. The aim of the present study is to analyze the biomechanics of fractures with a "butterfly" fragment, using a software that simulates the movement of the lines of force (and related iso-displacement points) that occur on the bone, when traumatic forces are applied on it. We have shown that the formation of the butterfly fragment derives from the application of three forces (compression, torsion and bending) with the bending force that acts by increasing the curvature of the long bone.

The fracture with a butterfly fragment has gained great interest in the 'field' of treatment, non-union problems, and forensic consideration. Biomechanics is an important source of information regarding the circumstances that 'led' to the fracture. Proper fracture interpretation may assist in identifying the right treatment or the right sequence of the events in the forensic perspective. In the clinical practice these kinds of fractures are observed more frequently in patients involved in road accidents and in pedestrian accidents (1, 2). Other causes of these fractures reported in literature are due to sports such as arm wrestling. Jacek K. et al. have analyzed 9 humeral shaft fractures in patients who practiced arm wrestling, in which rotational stress is high. In this study 5 of all fractures showed a pattern with a distal medial butterfly fragment (3). Also, low-velocity trauma can produce a butterfly shape fracture line (4, 5). From the biomechanical point of view the correct interpretation of causes and forces that lead to the formation of fractures with a third butterfly fragment is not yet clear. The aim of the present study is to analyze the biomechanics of fractures with a 'butterfly' fragment, using the software ANSYS which is a simulator of the movement of the lines of force (and related iso-displacement points) that occur on the bone, when traumatic forces are applied on it.

MATERIALS AND METHODS

The fundamental forces acting on a bone, including compression, transverse load, torsion and flexion, as the other forces, can be distinguished in their components, one along the axis of the bone and one perpendicular to it. The two major kinds of forces acting on a long bone are those that would tend to dislocate it in a linear direction (translation) and those that would tend to rotate it (rotation), around the center of the joint (center of rotation). When a force causes a rotation, it is a defined moment and has a distance, against which the force acts, determining the

Key words: butterfly fragment; biomechanical study; shaft; fractures

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rotation. Therefore, the moment is influenced not only by the intensity of the applied force but also by its distance from the center of rotation. The muscles can cause a rotation of the bone and so has a moment of rotation.

This study aims to investigate the forces acting directly on the bones using a software for simulating the mechanics of a traumatic event with the same compression and torsion characteristics that are frequently observed in the fractures examined, analyzing the forces in all directions of space.

This study has been conducted using the software ANSYS® that analyzed the development of lines of force and of the related iso-displacement points in the three-dimensionality of space, in a cylindrical solid (hypothetically ascribable to the geometric structure of a long bone). This solid was subjected to linear compression, torsion and bending forces, to simulate and recreate the ideal conditions for the development of the third butterfly fragment.

For simplicity, during all phases of the study, the cylindrical solid is oriented with its major axis along the z axis, on a plane identified by the respective x and y axes with an end fixed to a constraint. In the first session of simulations the iso-displacement lines were analyzed using a perfectly cylinder, then we tried to recreate a more realistic model, analyzing the iso-displacement points on a cylinder geometrically more similar to the macroscopic shape of a long bone, anatomically characterized by concavity and convexity.

RESULTS

The first five simulations analyzed the development of the iso-displacement points in a perfectly linear cylinder. In the first simulation in this cylinder we analyzed the development of the iso-displacement points along the y axis by applying a linear compression and a torsion forces on a contralateral constraint.

It can be observed that the greatest displacements are identified where there is application of force and gradually becomes smaller nearer to the constraint. It can also be noted that the iso-displacements points take a positive value in the right hemicylinder, and negative in the left hemicylinder; in particular, the points that have a greater value are those located in the lateral areas of the respective hemicycles (Fig. 1a).

In the second simulation the analysis is conducted considering the x axis by applying the same two forces. In this simulation the iso-displacement points

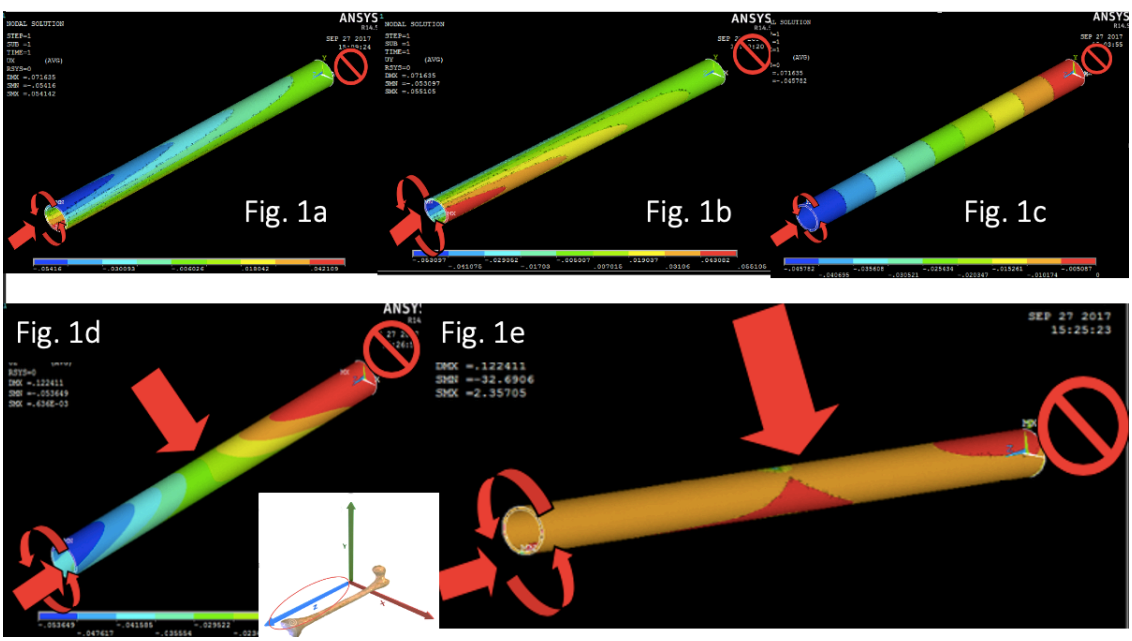

Fig. 1. *Analysis of the development of the iso-displacement points by applying a linear compression and a torsion forces on a contralateral constraint, in a linear cylinder.* ***a****): analysis conducted along the y axis;* ***b****): analysis conducted along the x axis;* ***c****): analysis conducted along the z axis;* ***d****): application in the midpoint of the cylinder of a bending force, orthogonal to the axis of the cylinder itself and with the analysis conducted conducted along the z axis;* ***e****): the same forces of the fig 1d, but analyzing only the component along the z axis, and no longer the iso-displacement lines.*

will have a similar behavior, but with an orientation of 90 ° with respect to each other, a consequence of the fact that the x and y axes are orthogonal to each other (Fig. 1b).

In the third simulation the analysis is conducted along the z axis. From the analysis of Fig 1c, we can clearly see the differences compared to the previously figures. The iso-displacement points had a more linear behavior with a negative shift; this is mainly due to the positioning of the cylinder which is oriented with its major axis along the z axis and on a plane between the x and z axis. It should be noted that the resultant of the forces applied distally to the cylinder causes a displacement of the most distal points of the cylinder towards the origin of the axes, which in this specific case corresponds to the region of application of the constraint. This is because only the compression force makes a movement while the torsional force has no influence on the movements along the z axis. Therefore, the points near the force application site had the greatest displacement and gradually approaches to zero, near the constraint.

In the fourth simulation the analysis is conducted along the z axis with the application of a linear compression force and a torsional force, with positioning of a contralateral constraint and application in the midpoint of the cylinder of a bending force with F (force) of 30 N and orthogonal to the axis of the cylinder itself. The addition of a further force, orthogonal to the axis of the cylinder, in the y / z plane, the iso-displacement points undergo a distortion similar for morphology to the lines observed in the previous simulation, but with a distortion that is greater at the point of application of the compression and torsion forces and at the constraint, while they remain unchanged at the point of application of the bending force. Therefore, we can deduce that the bending force has its greatest influence in the extreme points of the cylinder. Considering the trajectories of the areas represented, with the application of the bending force, the formation of a butterfly fragment begins to be defined (Fig. 1d).

In the fifth simulation we applied the same forces

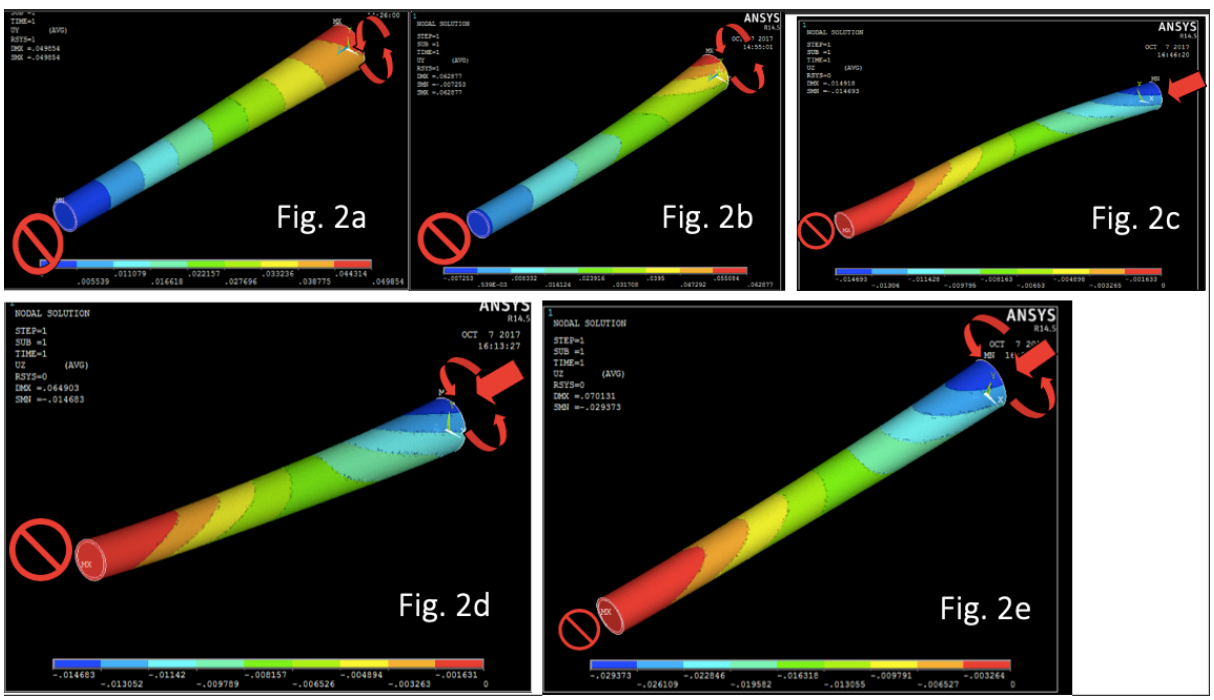

Fig. 2. *Analysis of the development of the iso-displacement points in a curved cylinder.* ***a****): analysis conducted along the y axis, with a radius of curvature (Dh) of 1 mm;* ***b****): radius of curvature has increased (5 mm);* ***c****) analysis conducted along the z axis;* ***d****) analysis conducted with a simultaneously application of an axial compression force and a counterclockwise torsion force with distal constraint;* ***e****) analysis conducted doubling the influence of the compression force.*

of the previous simulation and consider the resultant of the forces acting on the cylinder, analyzing only the component along the z axis, and no longer the iso-displacement lines. In this case it is necessary to highlight the transition point from forces with negative modulus to forces with positive modulus, since the fractures site occurs precisely at this point. From the figure we can see that the transition point is in the portion of the cylinder opposite the point of application of the bending force, showing the morphology of the third butterfly fragment (Fig. 1e).

At this point we analyzed the development of the iso-displacement lines in a cylinder that is not perfectly linear but in a curved cylinder. In the sixth simulation the analysis is conducted along the y axis with a radius of curvature (Dh) of cylinder equal to 1 mm, subjected to a torsional force with distal constraint. In this case, the radius of curvature of the cylinder influences the formation of iso-displacement lines, compared to a linear cylinder. These lines will be more visible in the areas closest to the point of application of the torsion force than in the areas closest to the constraint (Fig. 2a).

In the seventh simulation, the radius of curvature has increased (5 mm), making the cylinder more similar (compared to the previous simulation) in terms of morphology, to a long bone. The phenomenon showed in the previous simulation is amplified, in fact the iso-displacement lines become even more visible in the areas closest to the point of application of the torsion force. Therefore, it is as if the radius of curvature amplifies the effect of an external force directed along the concavity of the cylinder. Moreover, the curvature of the cylinder can, when a torsional force was applied, simulate the behavior of a linear cylinder with the application of the triple force previously described (Fig. 2b).

In the eighth simulation we examined the same cylinder, but along the z axis and applying axial compression force with distal constraint. In this case the curvature of a cylinder, at the same way, positively influences the development of the iso-displacement areas which graphically acquire the shape of a butterfly fragment (Fig. 2c).

In the ninth simulation we applied simultaneously an axial compression force and a counterclockwise torsion force with distal constraint (F = moment of torsion) (Fig. 2d). While in the linear cylinder there

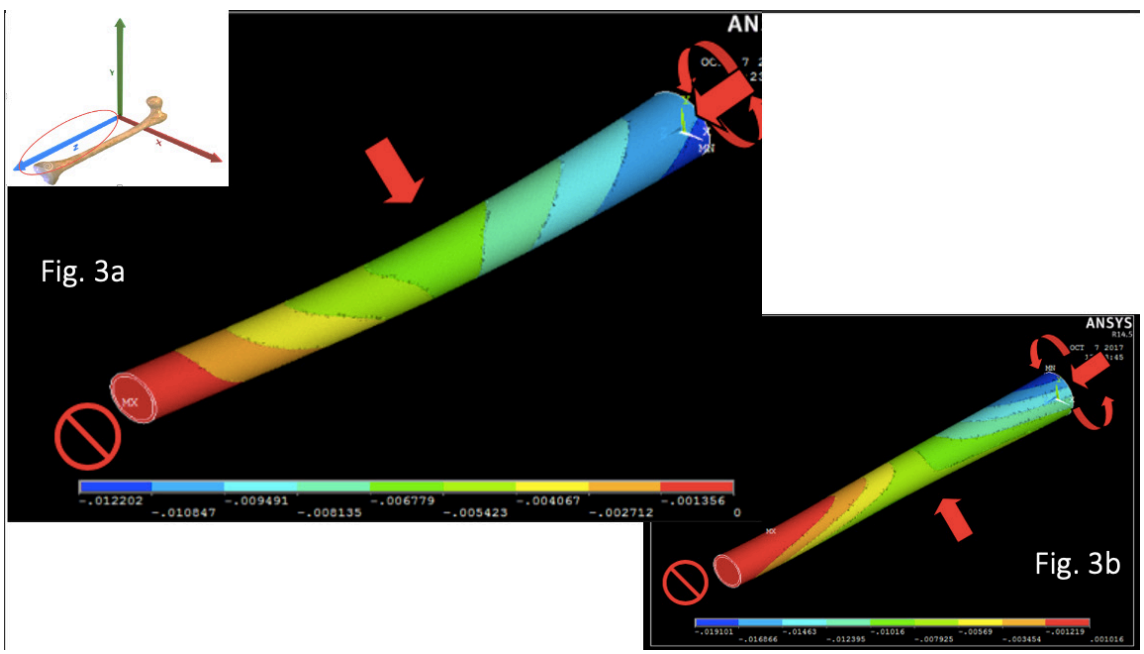

Fig. 3. *Analysis conducted adding a traction force (F = 10N) applied at the intermediate point of the curved cylinder.* ***a****): traction force is directed to increase the curvature of the long bone;* ***b****): traction force is directed to reduce the curvature of the bone.*

was a strong influence of the compression force, in the case of a curved cylinder the compression force has an influence on the development of the iso-displacement lines which gradually loses intensity in favor of the counterclockwise torsion force which becomes more influential in the development of the iso-displacement lines.

However, the development of the iso-displacement lines is more accentuated in this case, compared to the previous one, with a greater development of the areas underlying the iso displacement points where torsion and compression forces are applied and also at the constraint. It is precisely at the level of the constraint that the greatest variation of the iso displacement lines is observed, with even more evident formation of areas that take the shape of a butterfly fragment.

In the tenth simulation we have doubled the influence of the compression force compared to the torsion. The orientation of the iso displacement lines remains similar to the previous simulation; while the orientation of the areas underlying the displacement remains similar, we notice an increase of its intensity and the geometric conformation of a butterfly fragment become more evident (Fig. 2e).

In the last two simulations we added a traction force (F = 10N) applied at the intermediate point of the curved cylinder. In Fig. 3a this last force is directed to increase the curvature of the long bone, instead in Fig. 3b. it is directed to reduce the curvature of the bone. As showed in Fig. 3a the force directed to increase the curvature of the cylinder increases exponentially the development of the iso-displacement areas which are more deformed where the forces are applied and assuming a geometric shape hypothetically similar to the butterfly fragment.

DISCUSSION

The specific traumatic mechanism that leads to the formation of the butterfly fragment is debated in literature. The first hypothesis, presented in literature, was simple: the "butterfly shaped fracture" derived from a bending failure. When a bone is bent, the maximum compressive stress on the concave impact surface and the maximum tensile stress on the opposite convex surface is generated. Since cortical bone has a weaker tension than compression, failure begins with a tension opposite to impact (1). When the fracture reaches the compressed side, it has been suggested that the shear stresses exceed the shear strength of the bone and cause the fracture to divide along 45-degrees planes of the maximum shear (2).

Numerous researches show that bending forces create a more variable pattern of fracture than what was expected according the first hypothesis. Kress, in fact, reported that the bending forces applied to human femora and tibiae produces transverse, comminuted, oblique and butterfly fracture (6). Mariyam and Fenton showed no complete butterfly fractures in 3-point bending of anterior and posterior loaded human femora (2, 7). Moreover, Mariyam et all. confirmed the previous study of Reber demonstrating that long bone bending produces a variety of complete fracture patterns (2, 8). Haim C. et all. think that it should be a combination of forces to create a butterfly fracture. In fact, when they studied the major fracture lines, accompanied by the transverse line, they hypothesize that, in the moderate velocity impact, additional internal shearing forces developing in the bone can eventually create the butterfly pattern (5). The current study manages to analyze with a specific software the forces that can lead to the formation of fracture with a butterfly fragment. Starting from the analysis of the iso-displacement lines that are generated applying a single force on a curved cylinder, through various simulations, we get to define the conditions that are necessary for the formation of fractures with a third butterfly fragment.

On the basis of the different simulations performed and the analysis of the iso-displacement lines the resultant of the forces that determines the deformability as well as the fracture of a long bone should always be analyzed along the major axis of deformation of the bone itself.

The most important evidence is that the formation of the butterfly fragment in a curved cylinder (more similar to a real long bone) derives from the application of the three forces (compression, torsion and bending) with the bending force that acts towards increasing the curvature of the long bone.

Moreover, we have shown that as the only compression and torsional forces applied to a linear cylinder do not show any realization of iso-displacement lines, as well as, lines of force which lead to the hypothetical formation of the butterfly fragment. Other important evidence is that in a curved cylinder, its radius of curvature is directly proportional to the contribution of an external force directed along the concavity of the cylinder. Our study represents the first phase of the research. The next goal is to recreate, in cadaveric models, the conditions studied with this software.

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Adipose-derived stromal vascular fraction processed with different systems for the treatment of knee osteoarthritis: a pilot study on cell proliferation and clinical results

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In recent years, the interest in stromal vascular fraction (SVF) therapy for conservative treatment of osteoarthritis has grown significantly. This study aims to assess three different processing systems (micro-fragmentation, filtration, or slow centrifugation) in terms of cell proliferation *in vitro* and clinical results of intraarticular injections for the treatment of knee OA. From December 2017 to June 2018, 25 procedures were performed using three different systems. A considerable improvement of the clinical condition in almost all patients already one month after the treatment with a stable effect at 6 and 12 months was recorded. Patients treated with SVF, obtained by the micro-fragmentation system, had better outcomes one month after the treatment with a mean improvement of the symptomatology higher than that found in patients treated with the filtration or slow centrifugation system. The SVF product from the same system had a higher cell proliferation capacity *in vitro*.

Mesenchymal Stem Cells (MSCs) have trophic, mitogenic, anti-fibrotic, antiapoptotic, immunomodulatory and antimicrobial effects through the release of biologically active factors which can produce significant changes in the cellular environment (1, 2). About 2% of the cells contained in the adipose tissue are MSCs, compared to the 0.02% included in the bone marrow. Consequently, adipose tissue is a suitable source of MSCs, and its harvesting is related to a lower donor site morbidity (3, 4). MSCs derived from adipose tissue are defined as adipose-tissue-derived stem cells (ADSCs).

ADSCs are isolated in the aqueous fraction derived from the lipoaspirate material, and this fraction is known as stromal vascular fraction (SVF). It is mainly composed of ADSCs, endothelial progenitors cells (EPC), endothelial cells (EC), macrophages, smooth muscle cells, lymphocytes, pericytes, and pre-adipocytes (5). In recent years, the interest towards the application of SVF as a therapy for musculoskeletal disorders has grown significantly, especially as a potential option in the conservative treatment of osteoarthritis (OA) (6-9).

Different systems are available for the processing of lipoaspirate and the production of SVF (10). The function of these devices is to purify the lipoaspirate sample from oily and hemorrhagic fractions, minimizing the risk of complications and maximizing the biological yield for subsequent grafting (11, 12). However, few studies compared the efficacy of the different processing devices. This study aims to assess three different processing systems such as (1) micro-fragmentation, (2) filtration, or (3) slow centrifugation in terms of cell proliferation in vitro and clinical results of intraarticular injections for the treatment of knee OA.

Key words: Adipose-Derived Stromal Vascular Fraction, Knee, Osteoarthritis

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MATERIALS AND METHODS

Study population

The study received approval by the local ethics committee (Prot: 21.19 TS ComEt CBM). Inclusion criteria were symptomatic knee OA (stage II and III according to Kellgren-Lawrence scale) with a duration of symptoms longer than three months, unresponsive to non-injective conservative therapies (rehabilitation, cryotherapy, rest, NSAIDs), and without other injective treatments or previous surgery on the affected knee in the last 12 months. Patients with documented knee instability, osteochondral lesion, ipsilateral hip and ankle OA, rheumatoid arthritis, immunodepression, and severe general comorbidities (ASA grade 4) were excluded.

Harvesting of adipose tissue

The adipose tissue was harvested from the abdominal area. The subcutaneous tissue of the donor site was injected with 250 ml of Klein's solution (13) under sterile conditions. 10 minutes after the injection, around 40 ml of fat tissue were suctioned using a 2 mm multi-port small-hole cannula.

Processing of adipose tissue

The adipose tissue was then processed with three different methods.

1) Micro-fragmentation: The adipose tissue is injected into the processing unit previously filled with saline solution (0.9% NaCl). The processing unit, containing metallic spheres, is then shaken, exerting a mechanical reduction of the lipid clusters, and inducing cell activation. At the same time, the saline solution clears the sample from residues and blood components. At the end of the process, the lipoaspirate is separated from the saline solution and is pushed through a filter into a 10ml syringe.
2) Filtration: The lipoaspirate is injected into the processing unit through a portal and is washed with the addition of Ringer's lactate solution and is then purified, passing through a membrane. At the end of the process, the final product is then collected by suction with a 10ml syringe from the device.
3) Slow centrifugation: The lipoaspirate is injected into the processing unit previously filled with saline solution (NaCl 0.9%). The sample is purified through the thrust induced by a mechanical rotor propeller. The final product is then collected by suction with a 10ml syringe from the device.

Intra-articular Injection

Knee injection was performed with a superolateral approach under sterile conditions in the operative room immediately after the harvesting procedure. An average of 7 ml of the product was injected with a 20-g needle.

Tissue fraction, separation, and cell isolation

For each procedure, 1 ml of SVF product was collected, and 3 ml of PBS added to isolate the cells and centrifuge at 300 g for 5 min at room temperature to separate the tissue into the following layered fraction: blood/saline fraction in the inferior layer, fat tissue fraction in the intermediate layer and oily superior layer. The purified fat fraction separated was washed with PBS and then digested with 2mg/mL collagenase (Worthington) for 1h at 37°C under gentle agitation. Enzymatic digestion was blocked by adding 4 mL of fetal bovine serum. The digested tissue was filtered through a 70-μm cell strainer (Corning) to remove residual tissue. The cell suspension obtained was centrifuged at 200 g for 5 min. The cell pellet was used to assess cell proliferation. All the cells were seeded into T25 tissue culture flasks with Dulbecco's modified eagle medium; supplemented with 10% fetal bovine serum, 15% penicillin, and 1% streptomycin; and incubated in a humidified atmosphere at 37°C, with 5% CO_2. After 24h, the culture medium was replaced to remove non-adherent cells.

Cell proliferation

The cell proliferation of the isolated cell populations was measured using the trypan blue exclusion assay. Trypan blue staining (Abcam) was performed according to the manufacturer's instructions. From each sample, cells were collected after trypsinization (Corning) and were subsequently

counted using a Bürker's chamber. Cell count was executed weekly, for a total of 4 weeks of culture; the assay was performed in triplicate for each donor patient.

Clinical evaluation

Patients were clinically evaluated pre-operatively and at 1, 6, and 12 months after the procedure using a numeric Pain Rating Scale (NPRS) and the Western Ontario & McMaster Universities Osteoarthritis Index (WOMAC).

Statistical analysis

The entire statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, Inc. CA, US). The data are presented as median (interquartile range) in a non-Gaussian distribution and compared with each other by the Friedman test for paired data or by the 2Way ANOVA test for unpaired data.

RESULTS

From December 2017 to June 2018, 25 procedures were performed in 25 patients (8M, 17F) with a mean age of 60.5 years (SD=15) and a body mass index (BMI) of 25.6 (SD=5.5). The lipoaspirate was processed using a (1) micro-fragmentation system in 10 patients, (2) a filtration system in 8 patients, and (3) a slow-centrifugation system in 7 patients. None of the patients experienced local or systemic complications related to the operating procedure.

Cell proliferation

Regarding the evaluation of cell count and proliferation, the results were extrapolated from the institutional database. At the four weeks' time point, the micro-fragmentation system obtains an SVF sample with a cell count of 8297.5±2350.8: it was a higher cell proliferation compared to the other two methods, although not statistically significant. At the same time point, the cell count of the lipoaspirate was 5691.5±1694.8 when obtained by the filtration system and 5187.8±877.1 when obtained by slow centrifugation (Fig. 1).

However, it is important to note that at the T1 time point, the number of cells of samples treated with the filtration and slow-centrifugation systems was higher than the micro-fragmentation one.

Clinical outcomes

The collected data indicate a considerable improvement of the clinical condition in almost all

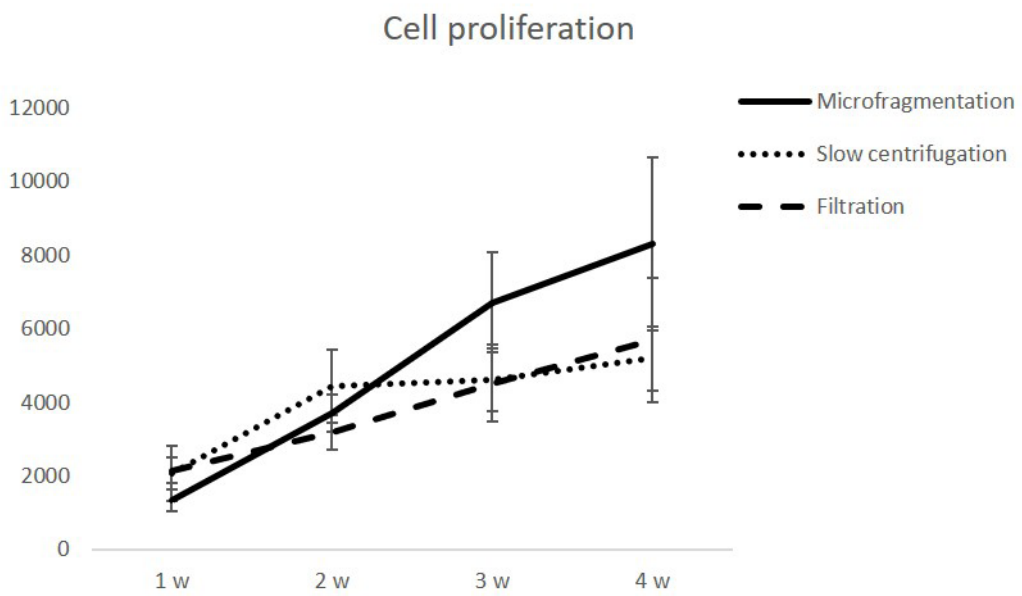

Fig. 1. *Graph showing cell proliferation of different systems analyzed.*

patients already at one month after treatment (for both values, ρ <0.0001 with Friedman test). The NPRS index before treatment indicates a mean value of approximately 7.96±1.19 while at the one-month follow-up, post-treatment indicates a mean value of about 5.07±1.99. The WOMAC index before treatment indicates a mean value of about 52%±12% while at the one-month follow-up, after treatment indicated a mean value of about 32%±15%.

After the improvement of the first month, the collected data indicate the stability of the clinical condition at the six and twelve-month control. The NPRS index at the sixth month follow-up, post-treatment indicates a mean value of approximately 4.59±1.9 while at the twelvth month follow-up, post-treatment indicates a mean value of about 4.81±2.05 (ρ <0.0001 with Friedman test). The WOMAC index at six months of follow-up post-treatment indicates a mean value of approximately 31%±13% while at the twelfth month follow-up, post-treatment indicates a mean value of about 33%±16% (ρ <0.0001 with Friedman test) (Fig. 2).

Micro-fragmentation NPRS index before treatment showed a mean value of approximately 7.75±1.75 while the NPRS index after treatment at the one month follow-up, showed a mean value of about 4.5±2.27, at six months, the follow-up post-treatment, indicates a mean value of approximately 4.25±2.34, while at the twelfth month follow-up, post-treatment indicated a mean value of about 4.33±2.27 (ρ = 0,0004 with Friedman test). The WOMAC score moved from a mean value of about 52%±14% pre-treatment to 31%±18% at one month, after treatment, at six months of follow-up post-treatment indicated a mean value of approximately 30.5%± 17.53% while at the twelfth month of follow-up, post-treatment indicates a mean value of about 29.75%±19.81% (ρ = 0.0004 with Friedman test).

The filtration system group for NPRS moved from 8.25±1,03 to 5.37±2.06 at one month after treatment, at the sixth month follow-up, post-treatment, indicates a mean value of approximately 4.75±1.75 while at the twelfth month of follow-up, post-treatment indicates a mean value of about 5±2.07 (ρ = 0,001 with Friedman test). The WOMAC index in the same group increased from 56%±12.6% to 35%±13.6% at one month of follow-up, at the sixth month of follow-up, post-treatment indicates a mean value of approximately 32.38%±10.6% while at the twelfth month follow-up, post-treatment, indicates a mean value of about 35.88%±12.81% (ρ = 0.0026 with Friedman test). The slow-centrifugation system

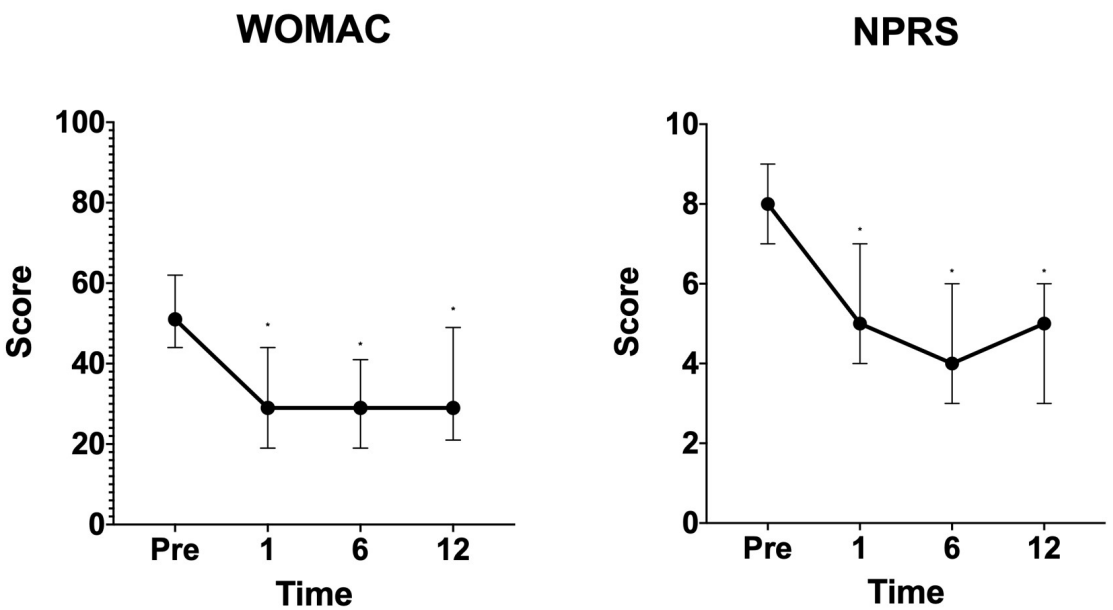

Fig. 2. *Linear graph of WOMAC and NPRS index before (pre) and after operation. *=r<0.0001.*

group recorded a mean NPRS index before treatment of about 8±1.16 and a mean post-treatment value of 5.71±1.25 at one month after treatment, at the sixth month follow-up, post-treatment, indicates a mean value of approximately 5±1.29 while at the twelfth month of follow-up, post-treatment indicates a mean value of about 5.43±1.71 (ρ = 0.0062 with Friedman test); the WOMAC index before treatment indicates a mean value of about 49%±11% while the WOMAC index after treatment at one month of follow-up indicates a mean value of about 32%±13% at one month after treatment, at six months of follow-up post-treatment, indicates a mean value of approximately 31.86%±12.05% while at twelve months of follow-up post-treatment indicates a mean value of about 34.71%±14.66% (ρ = 0.0054 with Friedman test). Detailed results are summarized in Fig. 3. ADSCs processed with the micro-fragmentation system resulted more effective in reducing the pain and improving joint function at one month, six months and twelve months after treatment, than that found in patients treated with the filtration or slow centrifugation system (ρ>0.05 with 2Way ANOVA test) (Fig. 3).

DISCUSSION

Degenerative joint diseases are an important and growing cause of disability and a socio-economic burden (14). Lacking in effective treatment, the knee OA remains a challenge for modern orthopedic surgery (15). Numerous conservative treatments have been proposed for pain control and functional improvement of patients aimed at slowing the progression of the disease (16, 17)notably OA. Conservative treatments such as physical therapy, pharmacological treatments (analgesics, steroidal and non-steroidal anti-inflammatories, opioids), hyaluronic acid and PRP, used for focal or diffuse lesions (18-21).

Advances in regenerative therapies and innovative MSCs technology offer a unique opportunity to treat OA appropriately and prevent the joint degeneration process.

In recent times, MSCs and ADSCs have been, with increasing importance, some of the highlights of research in regenerative orthopedics (22). Although the effect of ADSCs in repairing the joint cartilage damage is now well established, the recent application of this treatment remains extremely interesting (23). The use of the modern one-step methods of processing ADSCs in the form of SVF has given a further boost to the evolution of this new frontier of regenerative therapy (11, 12). Our transversal observational study allowed us to extrapolate and compare different one-step intraoperative systems to obtain SVF containing ADSCs.

The comparison of the indexes extrapolated from the questionnaires for the evaluation of the clinical condition indicates an improvement of the symptomatology in almost all treated patients. Comparing the results of the different systems, patients treated with SVF, obtained by the micro-fragmentation system, had better outcomes at one month after treatment with a mean improvement of the symptomatology higher than that found in patients

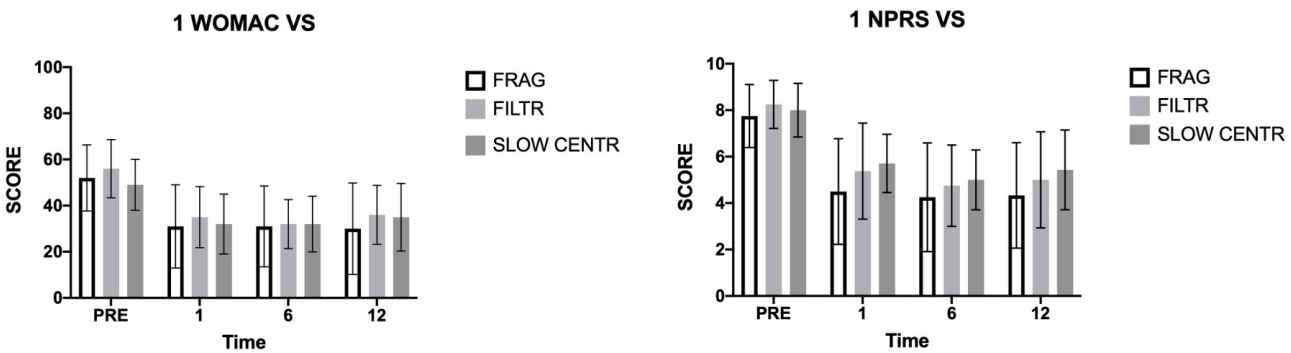

Fig. 3. *Bar graph of WOMAC and NPRS index before (Preop) and after operation: systems evaluation (Micro fragmentation, filtration and slow centrifugation).*

treated with the filtration or slow centrifugation system. The same system produces ADSCs with more pronounced cell proliferation. However, the samples treated with the filtration system and the slow centrifugation system have a higher cell count at the "T1" of our measurements. That may be due to the presence of microspheres in the processing chamber of the micro-fragmentation system, which generates mechanical stress and induces a reparative response from the treated cells.

Despite the results obtained, it highlights better results of the micro-fragmentation system compared to other systems, the present study has some limitations, such as the few samples analyzed, and only the quantitative, and not qualitative, analysis of the sample. These limitations have restricted our processing and comparison possibilities, giving us indicative but not significant results. Despite the results obtained they are particularly encouraging, yet our knowledge about the long-term efficacy of these treatments is still limited.

It is necessary to promote further studies on these recent therapeutic options, to investigate the issues that are still not documented in an exhaustive and irrefutable way. Prospective, randomized, double-blind studies, on a large sample of the population and with a longer follow-up, would give us fundamental answers for the correct understanding of the advantages, but also of the limits and contraindications, of this increasingly promising regenerative therapy.

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Megaprostheses in the treatment of complex fractures of the distal femur

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Total knee replacement (TKR) is a widely used procedure for the treatment of post-traumatic arthritis. This type of solution has also been used recently for the treatment of acute fractures around the knee, particularly in joints that already presented arthritis before the trauma. In case of complex fractures of the distal femur especially in older or very compromised patients, the use of a segmental implant can be an option of treatment in order to obtain a quicker recovery of the patient allowing early mobilization and weight bearing. The purpose of this paper is to present our experience with segmental TKR in the treatment of complex fractures of the distal femur highlighting the main problems associated with these conditions and focusing on the indications, principles of technique, tips, tricks and pitfalls of this procedure.

Complex distal femoral fractures and peri-articular knee fractures are difficult to deal with, especially in complex patients and in the elderly, because of poor bone quality, pre-existing arthritis, comminution and osteochondral damage at time of injury. A high 1-year mortality rate (22%) and significant decrease in function and quality of life have been noted in frail elderly patients who sustained supracondylar femoral fractures (1). Any intervention in this category of patients should ideally allow immediate fracture stability for early mobilization and early return to pre-injury functional level. Total knee replacement (TKR) is one viable option for this group of patients. There are good reasons, in the knee as much as for hip or shoulder fractures, for treating certain acute complex fractures using an arthroplasty, such as: significant symptomatic osteoarthritis prior to the fracture, fracture complexity, especially of its articular part, bone fragility is making fixation hazardous, and the need for early mobilization and the earliest possible resumption of walking in elderly or complex patients, in order to avoid the decubitus complications and the risk becoming bed-ridden.

The use of a distal femoral segmental prosthesis for the treatment of complex distal femoral fractures may be controversial for different reasons, but with limited indications and an accurate patient's selection can be a valid solution in order to obtain a quicker recovery and return to normal activities of daily living. The purpose of this paper is to present our results in the treatment of complex distal femoral fractures with a segmental implant in a very selected cohort of patients (Fig.1)

MATERIALS AND METHODS

Between January 2014 and December 2018, 15 distal femoral segmental prostheses were performed in 14 patients (one bilateral) with a complex distal femoral fracture. Essential demographics are summarized in table I.

Nine fractures were classified as AO 33.C3

Key words: total knee arthroplasty, segmental implants, fractures around the knee

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Table I. *Patient's essential demographics.*

Sex		Age	BMI
Females	Males		
10	3	74,6 (55-89)	26,2

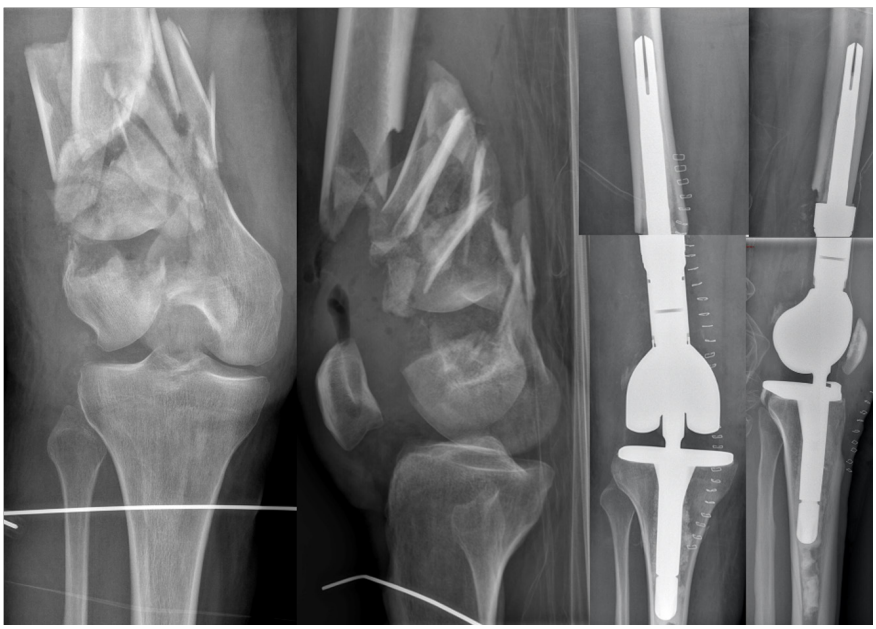

Fig. 1. *Treatment of a complex distal femoral fracture with Segmental implant.*

fractures and 3 as AO 33.C2 fractures, in 3 cases the segmental implant was used in order to revise a complex periprosthetic fracture of the distal femur with implant loosening (V3 B3 according to the Unified Classification for Periprosthetic Fractures UCPF) (2, 3). Ten surgeries were performed with the Zimmer Segmental System (ZSS Zimmer, Warsaw, IN) (Fig. 2) and 5 with the SMS distal femoral implant (Waldemar Link GmbH, Hamburg DE).

All the patients signed for informed consent before the surgery. All the procedures were performed by 2 experienced surgeons in the field of total knee arthroplasty (senior authors). No tourniquet was used. The rehabilitation protocol consisted of assisted full weight bearing from the first postoperative day and early free mobilization. After 15 to 30 days we allowed free walking without crutches. The mean follow-up was 36 months (range 12-70 months).

The choice of using a segmental implant was mainly related to the age of the patients (over 70 years old), the presence of an arthritic knee condition pre-existing to the trauma and a complex pre-existing general condition of the patient (ASA 3 or 4) requiring the possibility of an early mobilization and an immediate post-op weight bearing.

RESULTS

No patients were lost in follow- up, 3 patients died during follow up for causes not related to the implant and after having completed at least the 12 months follow-up visit. All the post-operative outcomes were evaluated at the last available follow-up. Results of clinical outcomes are summarised in table II.

There were 2 major complications: a 2-stage revision was needed in one case for a late deep

infection at 2 years after surgery; a revision for aseptic loosening after a trauma was needed for another patient at 5 years from index surgery.

DISCUSSION

Purpose of this study is to present the results of the use of a very specific type of implant in a very selected population of elderly or complex patients facing a complex trauma of the distal femur. The indication of using segmental implants even if in these very selected cases may be controversial and even for the authors the choice has been rigorously pondered as one can notice from the small number of patients involved in the study.

In general, the indication to perform a total knee replacement in the treatment of a fracture around the knee is controversial but the topic is gaining interest in recent times. A very interesting paper recently published by Parratte et al. (4) gives an overview on indications, surgical technique, tips and tricks on this topic, justifying the use of segmental implants in selected cases.

Same conclusions are presented by Benazzo et al. (5) in their paper describing the indications, technique and results of the treatment of acute cases and post-traumatic deformities. Malvyia et al. (6) showed good results in a small cohort of elderly patients treated with total knee replacement for fractures around the knee; in their study the authors were using different levels of constraint but in the majority of the patients a rotating hinged implant was used.

Kini et al. (7) introduced in their study the use of navigation in order to treat complex proximal tibial fractures with a TKA, concluding total knee arthroplasty should be considered a treatment option for acute

Table II. *Outcomes.*

Outcomes	Final Follow-up
ROM	85.8° ± 18°
KSS objective	71.7 ± 10
KSS functional	68.9 ± 12.2
HSS score	64.7 ± 3.5

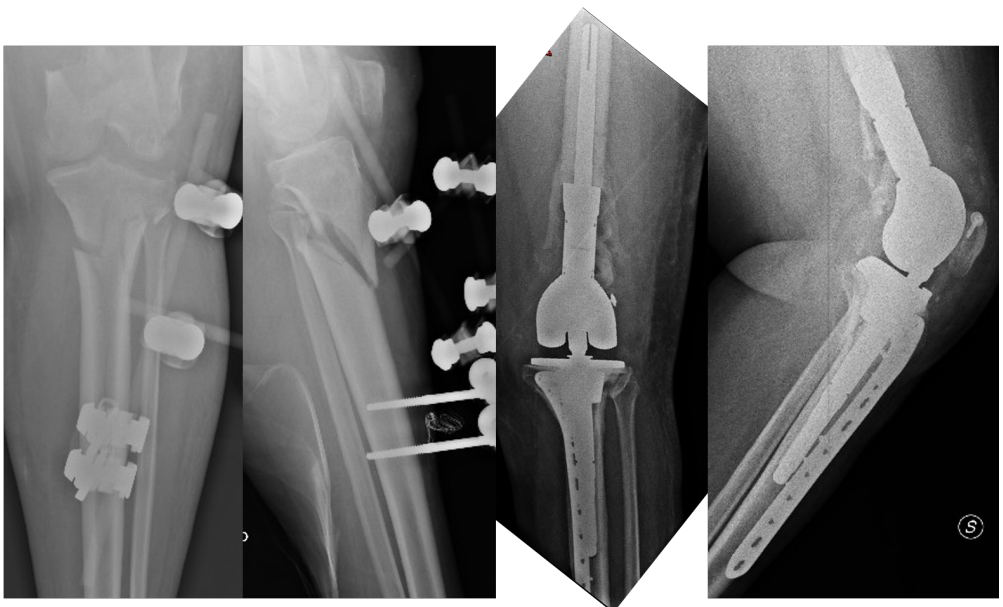

Fig. 2. *Treatment of a floating knee with segmental implant and associated proximal tibial open reduction and internal fixation (ORIF).*

upper tibial fractures in the elderly with coexistent knee arthritis and poor bone stock. They also found that computer-assisted navigation was helpful for restoration of mechanical axis and component positioning.

On the other side Boureau et al. (8) in their study, couldn't demonstrate that TKA could pre-vent loss of autonomy in elderly patients; despite surgical revision was less frequent than after osteosynthesis and resumption of weight-bearing was immediate, autonomy was still impaired. Mortality was comparable to other reports.

Finally, a recent review published by Meluzio et al. (9) showed that the use of knee megaprosthetic implants could represent a valid treatment option, aiming to reduce patients' immobilitazion and hospital stay and good clinical outcomes with low rate of complications were reported by all included studies. The authors also stress on the fact that literature is lacking in long-term outcomes and complications and that studies comparing knee prostheses and other types of surgical treatment (intramedullary nails, plate fixation system) are needed. They conclude that megaprostheses represents a viable treatment option in patients affected by DFFs (either acute, periprostethic or non-union) because they allow immediate weight-bearing, shorter hospital stay, a fast recovery of knee function and ADLs.

Our results are comparable with those presented in other studies; clinical outcomes are satisfying considering the type of implants used and the conditions of the patients. Concerning the 15% percent complication rate (2 cases) this is in line with the results presented in other studies. The infection was a late complication (at 2 years) and due to the patient's lifestyle; concerning the aseptic loosening, the patient presented after a traumatic event. The previous x-rays taken one year before were normal. The use segmental implants for complex distal femoral fractures may be an option in very selected patients to obtain immediate post-op weight bearing and early mobilization.

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New insights on metal allergy in total joint arthroplasty

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Metal allergy is an uncommon and not completely understood cause of failure in total joint arthroplasty (TJA). However, either immunopathology neither histologic studies clarified the mechanisms through which the metal ions could lead to the complications related to them. The lack of evidence around this topic also reflects the difficulties to diagnose the MRP in TJA. In fact, the diagnosis is generally based on the exclusion of other causes. Currently, skin-patch testing and lymphocyte transformation test (LTT) are being commonly used to investigate about metal hypersensitivity and a delayed type-IV hypersensitivity is the immuno-histologic response to metals involved in TJA loosening. A review of the recent publications about this topic has been made focusing on immunology, histopathology, and clinics to better understand a still debated topic in orthopedic practice.

Contact allergy to metals (i.e. nickel, cobalt and chromium) is a common disease in everyday life (1). Total joint arthroplasty (TJA) could be a relevant source of metal ions that leads to both cutaneous (i.e. dermatitis, vasculitis like reactions, urticaria) and non-cutaneous (i.e. pain recurrence, joint effusion, TJA loosening, reduced range of motion) manifestations. Particularly, a high rate of metal sensitization was observed in patients with complicated TJA (2). Immuno-response to metals is extremely frequent in case of metal-on-metal bearings used in hip arthroplasty, but several other sources of metal ions might be observed in TJA. The relevance of this topic is clearly demonstrated by the appearance of the terms "metal sensitivity" and "metal-related pathology" (MRP) as a diagnostic code in the Australian arthroplasty registry since 2012.

The cumulative incidence of MRP over a 15 year period was 3.9% and the MRP represents one of the principle reasons for revision in some specific types of TJA (i.e. hip resurfacing) (3). Considering that the sensitization to metals seems to be related also to the metal alloys and to the modularity of TJA (3), MRP represents a relevant issue to both the manufacturer and the orthopedic surgeon. However, the effective relationship between metal allergy (MA) and some TJA complications is still debated (3). The aim of the present study was to describe the red line that connects the immuno-response to the clinical manifestations to metal debris.

The immune response to metals

The immune response to a foreign body is characterized by a granulomatous reaction with the activation of several macrophages and only few lymphocytes (4). It has been observed that few patients develop a specific immune response against corrosion products such as Cobalt (Co), Chrome (Cr) or Nickel (Ni) ions (4, 5). Ni and Co are components of the TJA and known allergens in skin hypersensitivity. Patients with hip arthroplasties present a high degree of reactivity to metals probably

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due to a sensitizing effect of metal debris (6). Indeed, high levels of metal particles was detected in patients with both MA and failed implants (5). However, Christiansen et al, observed a significant increase in the levels of IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, GM-CSF, IFN-γ and TNF-α, in patients with aseptic loosening, without correlating it with MA (5).

On the other hand Hallab et al. (6), found a linear correlation between serum metal ion levels and an oligo-/monoclonal T-cell infiltrate around the implant, demonstrating an antigen-driven reaction that indicated a release of metal directly associated with MA in vivo (6). Allergies are hypersensitivity reactions characterized by an exaggerated immune response of the human body against substances. This concept implies a delayed type IV hypersensitivity (DTH), exemplified by the allergic contact dermatitis to metal ions (5). DTH is mediated by T cells and is independent from antibody reaction as opposite to type I-III responses.

In DTH an antigen-presenting cell (APCs) recognizes an allergen, binds and presents it to the T helper lymphocytes (Th1), activating an inflammatory response with the migration of cells to the inflammation site and promoting the production of antibodies from B cells. Metal ions are incomplete antigens (haptens) because of their low molecular weight and therefore to form an antigen capable of triggering a DTH must interact with proteins (5).

During the first phase (the sensitization) the APCs activate the Th1 that proliferate and produce long-life memory T cells capable of a stronger response when encountering the same antigen. The Th1-type inflammatory response might be dominant in the reaction of lymphocytes to metals in patients with TJA. In fact, a predominance of Th1 over the Th2 response was observed in aseptically loosened TJA, as demonstrated by the higher expression of the cytokines Th1-related than Th2 observed at the bone -prosthesis interface (6). The Th1 activity could be relevant in the pathogenesis of periprosthetic osteolysis. The particles engulfed by macrophages and the metal-protein complexes presented to lymphocytes lead to the production of both autocrine and paracrine factors that could lead to the periprosthetic bone resorption or bone ingrowth impairment (6).

It has been supposed that Th1, might be sensitized by the contact with metals debris from implants inserted before and after the joint replacement. This could lead to a reaction to the metal haptens of the subsequent TJA. Moreover, in association to this mechanism, the allergic response could also be related to naive lymphocytes activated by the metal particles released from the TJA itself.

Pro-inflammatory cytokines, like Il-1, IL-2, IL-6, INF-γ, and TNF, play a fundamental role in the DTH reaction to metals. Some cytokines, and especially IL-5, presents a significant increase after contact with Ni and therefore it was proposed as a marker for allergies (8). However, considering the high inter-individual and intra-individual variability of cytokine expression, the effective role of this cytokine in the identification of MA is debated.

IL-1β, IL-6, IL-8, TNF-α secreted by the activated macrophages and IFN–γ, together with the activation of the NF-κB pathway, affect bone formation and play an important role in the periprosthetic osteolysis as demonstrated by the high concentration in the tissues close to an aseptic loosened implant. These substances induce the differentiation of osteoclasts into mature cells, promotes osteoclast activation and osteoblast inhibition, with the result of an improvement in bone resorption. TNF-α is one of the first proinflammatory cytokine produced in response to wear particles and stimulates the production of both IL-1β and IL-6 from macrophages. The IL-6 seems to be important in the late phase of osteolysis, while IL-1β and TNF-α are critical mediators in the acute phase of inflammation. IL-8 is produced by macrophages as well as by osteoblasts, and osteoclasts. It has chemotactic properties on neutrophils and T cells and promotes the formation of osteoclasts.

Therefore, high levels of IL-8 in patients with aseptic loosening is probably related not only to the innate immune response, but also to the osteolytic process. IFN-γ is a produced by several immune cells and present both a pro and an anti-inflammatory activity. The prevalence of one or the other, depends on secretion levels and pathogenesis. IFN-γ activates macrophages stimulating the expression of both class I and class II major histocompatibility complex

(MHC), the production of cytokines (including IL-1, IL-6 and TNF-α) and surface molecules (e.g. ICAM-1, B7 and CD40) (6). The action of IFN-γ on bone resorption is unclear. Some studies showed a protective effect of on osteolysis, possible due to an inhibition effect on the early differentiation of osteoclasts, while others showed that IFN-γ promotes the formation of osteoclasts. Moreover, it seems that IFN-γ while having an inhibitory effect in the early stages of osteoclast differentiation, can improve their maturation in the late stages (5). Other cytokines involved in allergic responses have been detected in periprosthetic aseptic loosening (IL-4, IL-7, IL-9, IL-10) (7).

Recently a high concentration of both Th2 and Th17 had been observed in patients with MA in TJA (8). Therefore, a role of Th17 was hypothesized in the peri-implant inflammatory allergic reaction to Ni, making this reaction similar to that observed in autoimmune diseases (i.e. chronic inflammatory bowel disease) or inflammatory arthritis and morever, it was linked to the osteolytic process. Summer et al. showed that patients with a complicated TJA and a concomitant positivity to Ni patch, present a surprisingly high concentration of IL-17 and low levels of IFN-γ. On the other hand, patients with asymptomatic TJA with positive nickel patch test had a moderate IFN-γ expression without producing IL-17 (8). These contrasting observations further underline the extreme variability of the individual immune response and the need for further studies to better explain the mechanism of MA in TJA.

Histologic findings of periprosthetic metal reactions

The histological examination might help to clarify the different pathogenesis of implant loosening. An emerging concept in this field is the "Synovial-like interface membrane" (SLIM) (9). This is a term that indicates both the synovial tissue and the bone-implant interface membrane and could be studied to clarify the mechanism that led to implant loosening. For this purpose, a SLIM consensus-based classification had been developed, based on both histological and histochemical criteria (9). This classification differentiates seven patterns of adverse local tissue reactions to orthopedic implants.

Among these, SLIM type VI includes the "adverse local tissue reactions to implant materials (ALTRs) or to metallic debris (ARMD)". It represents an extension of the aseptic lymphocyte-dominated vasculitis-associated lesion (ALVAL) (9). It may be difficult to differentiate between particle toxicity and an allergy because of limited knowledge about mechanisms of reactions and properties of the particulate. Moreover, it must be considered that some of the described reactions might be mixed and overlapped. Three main histological patterns were found in SLIM type VI: (1) a macrophagic predominantly pattern with none or minimal lymphocytic response; (2) a mixed inflammatory pattern (both macrophagic and lymphocytic) with a variable presence of plasma cells, eosinophils, and mast cells and (3) a granulomatous pattern, that might be predominant, or associated with the mixed one .

Patterns 2 and 3 showed a population of T cells, or mixed T and B cells. The concomitant observation of a high concentration of mast cells and eosinophils, with or without formation of perivascular lymphocytic germinal centers, suggested a reaction to toxic wear with allergic/hypersensitivity response. The interpretation of the histological findings of a loosened implant should take into account the patients' clinical, radiological, microbiological, and allergology data (9). Moreover, data from the implants would be useful also to evaluate the corrosion patterns and wear particle characterization using transmission/scanning electron microscopy of the peri-prosthetic tissue.

However, high-grade lymphocytic infiltration in regardless the presence of tissue necrosis, suggest MA. In a histological evaluation of periprosthetic tissues obtained from patients with complicated metal-on-metal arthroplasty a cell-mediated DTH was observed (9). The samples showed a vasculitis with perivascular and intramural lymphocytic infiltration (CD3- positive T lymphocytes and CD20-positive B lymphocytes) of the postcapillary venules, swelling of the vascular endothelium, recurrent localized bleeding, necrosis, fibrin exudate and the accumulation of inflammatory macrophages, typical of the cell-mediated immune reaction (variable dimensions drop like inclusions) and in some cases eosinophilic granulocytes and mast cells (10).

However, a recent study on joint exposure to metal ions showed a higher increase in macrophages rather than lymphocytes (11) but can also be disseminated to remote organs. Periprosthetic tissues harvested during revision surgeries mainly reflect end-stage failure but may not adequately reveal initial biological reactions and systemic side effects. Therefore, primary reactions caused by metal particles and ions were investigated in an established murine model. Left knee joints in three groups, each consisting of ten female BALB/c mice, received injections of metal ions (MI). The inflammation cascade activated by wear particles might decrease the osteoblast activity while increase the osteoclast one. Moreover, an impairment in the mesenchymal stem-cell differentiation into functional osteoblasts might be observed. Finally, particles can inhibit the collagen synthesis by mature osteoblasts and induce their apoptosis (10). As a result, particles led to an increase in periprosthetic bone resorption and therefore to implant loosening.

The clinical manifestation of MA in TJA Clinics

Patients with MA to TJA may have a clinical presentation, quite difficult to interpret. The onset of symptoms might occur between two months and two years post-operatively (12). A comprehensive patients' history and clinical evaluation are needed (13). Patients might complain of both joint and skin manifestations, including local rash, erythematous papular lesions surrounding the skin incision, or throughout the entire body (14). The joint symptoms represent the greatest challenge for surgeons. Patients typically present joint effusion, stiffness or limited range of motion (1, 14), and very often this presentation is not distinguishable from a low-grade infection (14).

Therefore, the diagnosis of MA after TJA requires the exclusion of other painful TJA causes including aseptic loosening, infection, or implant malpositioning, crystal arthropathy or psychological disorders (8, 15). However, especially after total knee arthroplasty (TKA), a considerable percentage of patients had residual pain without a clear explanation (16). Standard x-rays are necessary to investigate on periprosthetic loosening, malalignment and component mal-positioning (15). Laboratory is important to evaluate that inflammatory markers (i.e. ESR, CRP) necessary to rule out infection (14, 16, 17, 26). Secondary level modalities (i.e. CT Scan) might be useful to further evaluate sings of component loosening or malpositioning (18). If these evaluations are inconclusive, preliminary exams for MA can be conducted (19).

Patch test (PT)

PT is the most commonly utilized test for suspected MA considering the low cost and easy to use (16). It is conducted placing cutaneous patches containing specific allergens and observing the development of a dermatitis, related to a delayed hypersensitivity at regular intervals (20). However, this test has some disadvantages including: the subjective nature of the immune response to an antigen, and the lack of a clear demonstration that the cutaneous response to an allergen reflects the process that occurs within the joint. In fact, the Langerhans cells (skin tissue APCs) could not be detected in the joint. Therefore, the use of patch test for the identification of a MA after TJA had been questioned.

Granchi et al. indicated that neither pre-operative either post-operative screening for MA are recommended, considering the lack of predictive value to both positive or negative results (21). Caicedo et al (22) demonstrated that a sensitivity to Ni or MA was not directly correlated to a hypersensitivity of implant materials. A recent study on 161 TKA, demonstrated the absence of a correlation between pre-operative positive PT and the rates of complications, reoperations or revisions (23). This observation was in contrast to that by Granchi et al, that reported a shorter median survivorship in patients with a positive skin patch (78 months with 120 months, respectively), although a direct relationship was not defined (24).

Lymphocyte Transformation Testing (LTT)

This test is performed by exposing blood lymphocytes and monocytes to a variety of metal salts and evaluating their proliferation during the following 7 days (22). Stimulation index represent the ratio of lymphocytes proliferation with and without allergen exposition (20). This test is an alternative to PT and eliminates the confounder of Langerhans

cells. Obviously, LTT has some disadvantages including: high costs, limited availability, inter-laboratory variability, lack of standardization, high rate of false-negative when the test processing is delayed, and the difficulties to maintain an appropriate sample (16, 19). Moreover, this test still needs to be validated (25). However, in a recent study comparing symptomatic and asymptomatic TJA, 17% of patients with a well-functioning implant had reactivity to Ni, while this percentage went up to 36% in case of a poorly functioning prosthesis. In a systematic review conducted by Granchi et al, the probability of a positive LTT was more than doubled for patients who had a loosened TJA compared with those with a stable one (21). Although promising, it is important to underline that a positive LTT in a patient with a poorly functioning arthroplasty does not imply a causality and, therefore, the diagnosis of MA in TJA is still to be demonstrated.

CONCLUSIONS

MA in TJA is a relevant cause of concern. The mechanism that underlines the immune response to metals are similar to those that control periprosthetic bone resorption. Particularly, DTH response seem to play a key role in both activities. However, the exact path that links the MA to its clinical manifestation is still unclear and further studies are needed to clarify the immune response that could be observed into a replaced joint. Considering the open questions around MA, its diagnosis as a causative factor of a complicated TJA is still questioned.

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The role of biophysical stimulation with pemfs in fracture healing: from bench to bedside

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Clinical biophysics investigates the relationship between non-ionizing physical energy and the human body. This narrative review aims to summarize the current evidence on the efficacy of PEMF-therapy in the promotion of fracture healing. The effectiveness of PEMFs has been deeply investigated in preclinical *in vitro* ed *in vivo* studies and level-I clinical studies. All these studies depicted only PEMF-devices with specific physical wave features - i.e. pulse shape, frequency and amplitude- could significantly promote bone repair. Moreover, the dose-response relationship was also defined in preclinical studies, thus providing the minimum exposure time needed in PEMF-therapy. PEMFs are currently employed in the management several bone injuries, including acute fractures at non-union risk, non-unions, osteotomies, stress fractures and osteonecrosis. Moreover, several ongoing studies are investigating the effectiveness of PEMFs on emerging clinical conditions, thus the indications to PEMF-therapy could potentially raise in future years.

Clinical biophysics is an emerging medical branch aiming to investigate the relationship between non-ionizing physical energy and the human body (1). A physical stimulus triggers a biological response by regulating specific intracellular pathways, thus acting as a drug (1). The efficacy of an electrical stimulus, however, depends on the specific features of its physical wave - i.e. pulse shape, frequency and amplitude- and on the exposure time, since only a specific type of wave could activate specific cellular targets (1).

Consequently, the concept of a "new soft-pharmacology" has been recently developed in clinical biophysics and, for each type of biophysical stimulation, a dose-response relationship has been described (1).

In the field of Orthopaedics and Traumatology, clinical biophysics focuses on both traumatic, orthopaedic and joint diseases. Consequently, three different subspecialties could be depicted, namely orthopaedic biophysics, traumatic orthopaedic biophysics and joint biophysics (2). Several types of electrical stimulation devices have received US FDA approval for orthopaedic application, including Pulsed Electromagnetic Fields (PEMFs), Combined Magnetic Fields (CMFs), Capacitive Coupling Electric Fields (CCEFs) and Low-Intensity Pulsed Ultrasound (LIPUS) (3). PEMF-therapy, however, currently plays a central role in the biophysical stimulation of fracture healing, both alone and as

Key words: Biophysical stimulation; PEMF; CCEF; fracture healing; bone marrow edema; non-unions; delayed unions; FRACTING score.

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an adjunct to the surgical treatment. CCEF-therapy, conversely, is mainly employed in the management of vertebral fragility fractures (VFFs) and the promotion of spinal fusions (3). This narrative review aims to summarize the current evidence on the efficacy of biophysical stimulation with PEMFs, in the stimulation of fracture healing.

Fracture healing physiology

Fracture healing is a complex physiologic process, relying on the crucial interplay between biological and mechanical factors, that finally results in the '*restitutio ad integrum*' of the injured bone (4-6).

The 'diamond concept' briefly remarks the four essential requirements strictly needed to obtain a physiological fracture healing. These requirements include a favourable mechanical environment and the presence of osteogenic cells, growth factors, and osteoconductive matrix and stimuli (4). The lack of one of the above-mentioned factors may lead to fracture delayed-union or non-union. Non-union is defined, according to the Food and Drugs Administration (FDA), as a fractured bone that has not shown progression towards healing over three consecutive months on serial radiographs (7, 8).

The "personality" of the injury, i.e. the fracture pattern, the soft tissue status and the patients' feature should be finally assessed. Therefore, several fracture-related and patient-related, either biological and mechanical factors, might interfere with the fracture healing process (9-12). Fracture-related factors include location and features of the fracture site, fracture line characteristics, type of fixation, quality of fracture reduction, bone quality and vascularization and soft tissue status (13). Patient-related factors include age, smoking status, comorbidities (diabetes, obesity, osteoporosis, alcoholism…) drugs assumption (nonsteroidal anti-inflammatory drugs, corticosteroids…) (3, 14).

PEMFs: mechanism of action

An electromagnetic field is a physical phenomenon resulting from the combination of an electric field and a magnetic field. The electromagnetic fields employed in clinical biophysics have a sinusoidal 76-Hz frequency and a magnetic flux density in the range between 0.1mT and 30mT (15).

The PEMF device acts by producing a magnetic field that, in its turn, induces currents to flow in nearby tissues (16). These induced currents mimic the natural electrical activities created within bones during movements, thus triggering a cascade of activities, from the cell membrane to the nucleus and on to the gene level, where specific changes take place (17).

PEMFs mechanism of action deeply studied *in vitro* in human bone marrow cells (hBMCs), osteoblastic cells (MC3T3-E1) and osteoblast-like cell lines (SAOS-2), involves the activation of the ryanodine-sensitive calcium channels in the sarcoplasmic reticulum(18). The resulting increased cytosolic calcium concentration triggers, on its turn, the calcium-calmodulin pathway, thus causing the upregulation of the genes of the TGF-beta/BMP superfamily (19). All these genes have a synergic effect in promoting the differentiation of mesenchymal stem cells (MSCs) into osteoblasts and the synthesis and bone extracellular matrix, thus facilitating the fracture healing process (15).

In vitro studies were also useful to identify, in stimulated cultured bone cells, the relationship between PEMF exposure times and the enhancement of cells proliferation (20).

Preclinical evidence in fracture healing

Several *in vivo* preclinical studies have reported positive effects of PEMFs in bone healing. In 1974, Bassett et al. showed, for the first time, PEMFs are effective in enhancing a fibula osteotomy repair, in a dog model (21). Moreover, these authors depicted stimulated bone presents physiological bone repair and improved biomechanical properties, compared with non-stimulated bone (21).

Further *in vivo* studies have focused on the study of the optimal PEMF signal features in bone repair, the kinematic of skeletal bone healing, the positive effects of PEMFs on the subchondral and epiphyseal bone deposition and the integration of both autologous osteochondral grafts (22).

Moreover, Fini et al. have evaluated, in a rabbit model, the effect of stimulation with PEMFs on the osseointegration of hydroxyapatite in cortical bone in rabbits (23).

Stimulated implants, inserted into the femoral cortical bone and treated for six hours per day for three weeks, were compared with non-stimulated ones. Interestingly, in stimulated animals, and improved hydroxyapatite scaffolds osseointegration, higher peri-implant bone maturity and better mechanical fixation were depicted (23).

From bench to bedside: the realization of a pemf device

In 1979, PEMFs were approved as a safe and effective treatment in the management of bone non-unions by the FDA. In recent years, pocket-devices have been produced, to make the stimulation sessions more comfortable, thus hopefully enhancing the patients' compliance.

In such a kind of device, the magnetic field is generated by a pair of circular coils of copper wire, placed opposite each other. The coils are powered by the generator system, that produces the input voltage of pulse.

The pulse duration of the signal is 1.3 ms and the repetition rate is 75 Hz, yielding a duty cycle of 1/10. The intensity peak of the magnetic field is 1.5 mT and the induced electric field, as detected with a standard coil probe (50 turns, 0.5 cm internal diameter of the coil probe, 0.2 copper diameter), is 0.07 mV/cm.

As previously stated, the exposure time is crucial for the PEMF-therapy effectiveness, consequently, the PEMF device, to significantly enhance bone healing should be used 8 hours per day, for at least 45-60-days (depending on the fracture and patient's features) (15).

Clinical evidence in bone repair

Based on level-I evidence studies, PEMFs are currently used in clinical practice in the treatment of osteotomies, acute fractures at non-union risk, non-unions, stress fractures and osteonecrosis (1). Borsalino et al. and Mammi et al. have demonstrated PEMFs effectiveness in the promotion of bone repair in intertrochanteric femoral osteotomies and tibial osteotomies, respectively (15).

In the management of osteonecrosis, PEMFs revealed effective both in the conservative management of hip osteonecrosis, in the early stages, or as an adjunct to core decompression and bone grafting (1). Massari et al. therefore, reported 53% of simulated patients (PEMFs used 8 hours/day for two months) complained no longer for hip pain at the two month follow-up and 26% of patients reported a significant pain reduction. Santori et al. on the other hand, reported PEMFs, used as an adjunct to core decompression and bone grafting, showed a success rate of 81% and 70% in patients with Steinberg stage II and III hip osteonecrosis, respectively.

Focusing on fracture healing, Fontanesi et al. reported a significant reduction of fracture healing time in stimulated patients with acute tibial fractures, compared with controls (15). Moreover, Faldini et al, in stimulated patients with femoral neck fractures undergoing cancellous screws fixation, observed an increase in the percentage of fracture healing, compared with controls (15).

The use of PEMFs in the management of non-unions is supported by relevant clinical evidence but is indicated only in presence of a valid mechanical environment, therefore the fracture alignment, the limb immobilization and the lack of a significant bone loss should be assessed and corrected, before considering PEMF-therapy. The success of PEMF-therapy in the treatment of non-unions (adequately managed from a mechanical point of view) ranges between 73% and 85%, based on fracture and patients' related factors and patients' compliance (1).

Cost-benefit analysis has recently justified early PEMFs use in fractures at non-union risk (15). Big efforts have been recently made by the orthopaedic surgeons' community to promptly identify fractures at non-union risk. For instance, the healing time of tibial fractures surgically managed could be reliably estimated using the FRACTING score, developed in an Italian multicentre Level-I clinical study (24). This score could be easily calculated, in daily clinical practice, by using the mobile medical app "FRACTING". In the presence of a FRACTING score predicting prolonged healing time, the use of PEMF-therapy should be useful to prevent the fracture non-union.

Future developments

Several studies are currently investigating the role of PEMFs in bone repair, therefore, in future years,

there could be increasing employment of biophysical stimulation in clinical practice. The ongoing studies are focusing on the role of PEMFs in the treatment of several musculoskeletal diseases, including bone loss after a forearm fracture (clinicaltrials.gov: NCT00067834); acute distal radius fractures (clinicaltrials.gov: NCT04287257); fifth metatarsal fractures non-union (as an adjunct; clinicaltrials.gov: NCT00586170); odontoid fractures (clinicaltrials.gov: NCT02281994) and maintenance of bone and muscle mass in patients who have undergone ACL reconstruction (clinicaltrials.gov: NCT03165318) (25).

PEMF-therapy is a valid tool to enhance bone repair, in fractures undergoing an adequate biological and mechanical treatment. The effectiveness of PEMFs has been deeply investigated in preclinical *in vitro* ed *in vivo* studies and level-I clinical studies.

PEMFs are currently employed in the management several bone injuries, including acute fractures at non-union risk, non-unions, osteotomies, stress fractures and osteonecrosis. Moreover, several ongoing studies are investigating the effectiveness of PEMFs on emerging clinical conditions, thus the clinical indications to PEMF-therapy, in orthopaedics and trauma clinical practice, could potentially raise in future years.

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