Protocol cover page

Title: PATTERN- *P*rediction *A*lgori*T*hm for regenera*T*ive medicine approach in kn*E*e OA: new decision-making process based on patient p*R*ofili*N*g (CO-2019-12370720 Prof. Kon BANDO DI RICERCA FINALIZZATA 2019)

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Date

CENTRE SIGNATURE – PRINCIPAL INVESTIGATOR

I have read this Protocol Amendment relevant to the study entitled "**PATTERN -Prediction AlgoriThm for regeneraTive medicine approach in knEe OA: new decision-making process based on patient pRofiliNg**" and I agree to conduct the study as detailed herein and in compliance with guidelines for Good Clinical Practice and applicable regulatory requirements. I will provide all study personnel under my supervision with all information provided by the Sponsor and I will inform them about their responsibilities and obligations.

ELIZAVETA KON
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Glossary of abbreviations

IEC	Indipendent ethics committee
ICH/ GCP	International Conference on Harmonisation (ICH) /Good Clinical Practice standard
MoH	Ministry of Health
BMAC	Bone Marrow Aspirate Concentrate
at-SVF	adipose tissue enriched in SVF
MSCs	Mesenchymal Stem Cells
OA	Osteoarthritis

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1 Summary

Title	***P***rediction ***A***lgori***T***hm for regenera***T***ive medicine approach in kn***E***e OA: new decision-making process based on patient p***R***ofili***N***g (CO-2019-12370720 Prof. Kon BANDO DI RICERCA FINALIZZATA 2019)
Investigator sponsor	Humanitas Research Hospital
Study coordinator	Prof. Elizaveta Kon
Protocol identifying number	
Protocol version date	
Background and rationale	Osteoarthritis (OA) is a degenerative joint disease characterized by chronic pain, degradation and loss of articular cartilage, osteophytes formation and different degrees of synovial inflammation [1]. OA has a strong impact on society in terms of life quality and economic burden, given its rapidly growing prevalence [2], in particular among the elderly [3], and its impact on daily activity [4]. Nowadays, most of the available conservative treatments, such as physical therapy, anti-inflammatory drugs and viscosupplementation, provide temporary symptoms relief but have no effect on the cause and progression of the pathology [5]. Surgical joint replacement represents a successful and durable option for severe OA, while in early stages the success of this approach is variable and the outcomes are often unsatisfactory. This is especially the case in the challenging subset of younger OA patients with high functional demands yet limited indications for invasive surgical treatments, the so called "not ready for metal" patients [6]. Recently, the issue has even extended to older but still active patients who expect to maintain a high activity level and want to postpone or avoid metal reconstruction [7]. Mesenchymal stem cells (MSCs) have emerged as a durable and effective conservative treatment option for OA [8]). These are tissue cell-rich concentrates, that demonstrated immunomodulatory activities in several in vitro and in vivo studies [9], in particular in orthopaedics [10,11]. Then, "minimal manipulation" methods for the intraoperative production of tissue cell-rich concentrates has become a popular

(and sometimes abused) strategy in clinical practice. In particular, bone marrow aspirate concentrate (BMAC) and adipose tissue enriched in SVF (at-SVF), namely "orthobiologics", emerged as convenient and promising sources, with a high safety profile and positive shortterm clinical outcomes [12,13] and became very popular in clinical practice.
Despite the growing evidence concerning the use of orthobiologics, different preparation and administration methods and the lack of a meaningful data collection do not allow for a clear comprehension of the real efficacy of these treatments, also resulting in the lack of specific indication for each patient. In fact, a percentage of about 20-30% of non-responders to orthobiologics is reported in several studies [14-17], but all the Authors failed to identify the possible causes so far. One of the reasons can be the use of inefficient delivery methods. Although the most common way to deliver regenerative medicine products is intra-articular injection, more recently also the subchondral bone of OA patients has been demonstrated to undergo relevant pathological changes, including microcracks and structural defects, vascularization of channels, nerve growth, and a progressive replacement of the subchondral marrow with fibro-neurovascular mesenchymal tissue [18]. Given this evidence, intraosseous (bone-cartilage interface) injections of biological products can represent a promising approach [19].

A 4-arms randomized clinical trial in 240 patients affected by knee OA KL 2-3. Treatment groups: 1) intra-articular injection of BMAC, 2) intra-osseous and intra-articular injection of BMAC, 3) intra-articular injection of adipose tissue enriched in SVF (at-SVF) , 4) intraosseous and intra-articular injection of at-SVF.

Population and patient selection criteria

Inclusion criteria:

1. Patients aged 18-75;
2. Patients affected by knee OA KL 2-3;
3. Patients understanding the nature of the study and providing their informed consent to participation;
4. Patients willing and able to attend the follow-up visits and procedures foreseen by study protocol;
5. Body mass index (BMI) ≤ 40 kg/m2;

6. Ability to provide written informed consent and can understand and comply with the requirements of the study.

Exclusion criteria:

1. Patients with known inflammatory diseases at the time of enrolment.
2. Patients who are not allowed to undergo the study procedures involving imaging (X-rays, MRI) based on Investigator's judgement.
3. A history of local anaesthetic and anticoagulant drug allergy;
4. Clinically observed active infection in the index knee joint or skin disease/breakdown or infection in the area of the planned injection site of the index knee;
5. Major surgery (e.g. osteotomy) of the index knee within 12 months prior to screening;
6. Minor surgery (e.g. shaving or arthroscopy) of the index knee within 6 months prior to screening;
7. Patients who received intra-articular injection of corticosteroids, PRP or HA within the previous 3 months;
8. Use of systemic immunosuppressants within 6 weeks prior to screening;
9. Patients with a history of invasive malignancies (except non-melanoma skin cancer), unless treated with curative intent and with no clinical signs or symptoms of the malignancy for 5 years;
10. Patients who are participating or have participated in other clinical studies within the 30 days before the study enrolment.
11. Women who are pregnant or breast-feeding or who wish to become pregnant during the period of the clinical investigation and for three months later.

Study design and study duration

A Multicenter, open-label, randomized, four-arms clinical investigation. The Patients enrolled in this clinical investigation will undergo a scheduled surgery for the treatment of OA KL 2-3 grade. After the enrolment the patients will be assigned to 4 treatment groups: 1) intra-articular injection of BMAC, 2) intra-osseous and intra-articular injection of BMAC, 3) intra-articular

	injection of at-SVF, 4) intraosseous and intra-articular injection of at-SVF. Medical history, clinical and radiological features as well as a serum samples, will be collected for each patient at baseline, together with cell count of each injected product. 1) Determination of the most effective regenerative treatment (from the 4-arms clinical trial) for knee OA KL 2-3. Identification of responders and non-responders. The primary efficacy endpoint is the mean change from baseline to 12 months in the average subscales scores of Pain and Symptoms in the Knee injury and Osteoarthritis Outcome Score (KOOS) questionnaire for each arms. KOOS subscales scores of Pain and Symptoms will be aggregated and averaged as primary outcome. 2) Evaluation of the clinical outcomes at 3/6/12 months follow-up, by KOOS, VAS and IKDC questionnaires. Radiological evaluation at 6 months
Objectives	Classification of responders and non-responders based on the KOOS improvement (difference between KOOS at 6 months and baseline >10 or <10, respectively). Collection of data and serum samples from all patients. 2) Patient profiling through high-throughput proteomic analysis on serum samples and validation of the markers differentially expressed in responders and non-responders 3) Development of an algorithm for the discrimination of the most appropriate treatment based on the patient's specific characteristics collected during the project. A diagnostic kit for the evaluation of the identified relevant biomarkers will be developed and patented by the PI in order to support the algorithm future application
Statistical methods, data analysis	Sample Size Calculation: 60 patients will be enrolled in each study group, including a 15% dropout rate. This will allow to record a significant difference of 10 points in KOOS scale among the groups (α=0.05, β=0.1, expected standard deviation=15, t test for independent variables).

Data analysis:

Differences between baseline and 6-month evaluations within groups: paired t-test or Wilcoxon matched paired test.
Further analysis: mixed model repeated measures (MMRM) with an unstructured covariance and containing baseline KOOS together with treatment group.
Proteomics data: MaxQuant-Andromeda software suite and differences in biomarkers levels will be analysed by Perseus software.
P-values <0.05 will be considered statistically significant.

Study time table

months 0-18
- 240 patients treated
- complete database of anamnestic, radiological, clinical data;
- collection of all serum samples.

months 3-30
- Database containing clinical evaluations for all patients at 3, 6, 12 months;
- collection of radiological observations at 6 months.

months 20-24
- List of responder and non-responder patients in each group.

months 20-30
- list of biomarkers differently expressed in responders and non-responders

months 26-32
- list of validated biomarkers differently expressed in responders and non-responders

months 24-34
- proficient algorithm for the choice of the most effective regenerative medicine treatment based on patient specific profile

months 28-34
- diagnostic kit for the evaluation of relevant biomarkers identified during the project

months 35-36
- closing meeting

2 Background and introduction

Regenerative medicine has emerged as a promising approach for the treatment of Osteoarthritis (OA), but the lack of independent studies giving clear indications for specific treatments with bone marrow- or adipose-derived stem cell concentrates and the percentage of non-responder patients makes its application in clinical practice confusing. The project aims to provide clinicians with a proficient decision-making algorithm to ameliorate indications in regenerative medicine.

Patients affected by knee OA Kellgren-Lawrence (KL) 2-3 will be treated by intra-articular and/or intra-osseous injection of bone marrow- and adipose tissue-derived stem cell concentrates. Medical history, clinical and radiologic data will be collected from all patients along with a high-throughput analysis of serum biomarkers, they will be used to build an algorithm for the selection of the most effective treatment for each patient. Ultimately, a diagnostic kit will be developed based on the identified biomarkers.

3 Rationale of the study

Osteoarthritis (OA) is a degenerative joint disease characterized by chronic pain, degradation and loss of articular cartilage, osteophytes formation and different degrees of synovial inflammation [1]. OA has a strong impact on society in terms of life quality and economic burden, given its rapidly growing prevalence [2], in particular among the elderly [3], and its impact on daily activity [4].

Nowadays, most of the available conservative treatments, such as physical therapy, anti-inflammatory drugs and viscosupplementation, provide temporary symptoms relief but have no effect on the cause and progression of the pathology [5]. Surgical joint replacement represents a successful and durable option for severe OA, while in early stages the success of this approach is variable and the outcomes are often unsatisfactory. This is especially the case in the challenging subset of younger OA patients with high functional demands yet limited indications for invasive surgical treatments, the so called "not ready for metal" patients [6]. Recently, the issue has even extended to older but still active patients who expect to maintain a high activity level and want to postpone or avoid metal reconstruction [7]. Mesenchymal stem cells (MSCs) have emerged as a durable and effective conservative treatment option for OA [8]).

These are tissue cell-rich concentrates, that demonstrated immunomodulatory activities in several in vitro and in vivo studies [9], in particular in orthopaedics [10,11].

Then, "minimal manipulation" methods for the intraoperative production of MSCs-containing cell concentrates has become a popular (and sometimes abused) strategy in clinical practice.

In particular, bone marrow aspirate concentrate (BMAC) and adipose tissue enriched in SVF (at-SVF), namely "orthobiologics", emerged as convenient and promising sources, with a high safety profile and positive shortterm clinical outcomes [12,13] and became very popular in clinical practice.

Despite the growing evidence concerning the use of orthobiologics, different preparation and administration methods and the lack of a meaningful data collection do not allow for a clear comprehension of the real efficacy of these treatments, also resulting in the lack of specific indication for each patient. In fact, a percentage of about 20-30% of non-responders to orthobiologics is reported in several studies [14-17], but all the Authors failed to identify the possible causes so far. One of the reasons can be the use of inefficient delivery methods. Although the most common way to deliver

regenerative medicine products is intra-articular injection, more recently also the subchondral bone of OA patients has been demonstrated to undergo relevant pathological changes, including microcracks and structural defects, vascularization of channels, nerve growth, and a progressive replacement of the subchondral marrow with fibro-neurovascular mesenchymal tissue [18]. Given this evidence, intraosseous (bone-cartilage interface) injections of biological products can represent a promising approach [19]. Based on these lines of evidence, there are an enormous knowledge gap and a significant unmet need to profile patients before treatments to predict response and optimise treatment allocation. Thus, a specifically-aimed clinical study which includes the complete patient profiling and assessing the best indications for the use of orthobiologics in OA is compelling.

4 Objectives of the study

4.1 General objectives

Determination of the most effective regenerative treatment (from the 4-arms randomized clinical trial) for knee OA KL 2-3.
Identification of responders and non-responders.

4.2 End-points

4.2.1 Primary endpoint

The primary efficacy endpoint is the mean change from baseline to 12 months in the average subscales scores of Pain and Symptoms in the Knee injury and Osteoarthritis Outcome Score (KOOS) questionnaire for each arms.
KOOS subscales scores of Pain and Symptoms will be aggregated and averaged as primary outcome.

4.2.2 Secondary endpoint

- Patient profiling through collection of clinical, radiological and anamnestic data, and through a high-throughput proteomic analysis of serum samples and validation of the markers differentially expressed to identify possible specific characteristics of patients responders and non-responders to each treatment.
-Whole-Organ Magnetic Resonance Imaging Score (WORMS) at 6, 12 months compared to baseline;
- Presence/occurrence of structural pathological features detected by MRI at 6, 12 months compared to baseline;

WORMS

The Whole-Organ Magnetic Resonance Imaging Score (WORMS) is the most used semi-quantitative scoring system in knee OA clinical trials and is well validated. Every structure will be quantified by trained readers according to a well-established scale (Peterfy et al., 2004). This system will allow evaluating treatment efficacy in the different compartments and structures of the joint, and thus quantify focal improvements/degradations.

The technique will be optimized to reduce time acquisition to ensure subject comfort and will not require any intravenous injection contrast prior to image acquisition.
Scores will be reported for the 14 subregions of the knee as defined by Peterfy et al in 2004:
• Medial Patella;
• Lateral Patella;
• Medial Femur (anterior, central and posterior);
• Lateral Femur (anterior, central and posterior);
• Medial Tibia (anterior, central and posterior);
• Lateral Tibia (anterior, central and posterior);
• S zone (when appropriate).

Features to be scored:
• Cartilage;
• Bone Marrow lesions;
• Kysts;
• Attrition;
• Osteophytes;
Scores will also be reported for:
• Meniscus (medial and lateral; anterior, central and posterior regions, extrusion);
• Ligaments;
• Loose bodies;
• Synovitis;
• Bursal collections.
The semi-quantitative WORMS and its 14 features (articular cartilage integrity, subarticular bone marrow abnormality, subarticular cysts, subarticular bone attrition, marginal osteophytes, medial and lateral meniscal integrity, anterior and posterior cruciate ligament integrity, medial and lateral collateral ligament integrity, synovitis/effusion, intraarticular loose bodies, and periarticular cysts/bursitis) will be evaluated in 14 subregions (patella/femur/tibia) of the knee. T2 relaxation time will be evaluated in patella, femur and tibia subregions and in the following cartilage sub-regions medial tibia, medial weight bearing femur, medial trochlea, lateral tibia, lateral weight bearing femur and lateral trochlea. Cartilage volume (mm3), thickness (mm), and bone curvature were reported for femur, tibia, patella, and for the following cartilage subregions: medial tibia, medial weight bearing femur, medial trochlea, lateral tibia, lateral weight bearing femur and lateral trochlea. Any other additional structural pathological feature detected by MRI will be collected and evaluated.

5 Patient selection criteria

The following criteria will be applied to all the patients.

5.1 Inclusion criteria

1. Patients aged 18-75
2. Patients affected by knee OA KL 2-3.

3. Patients understanding the nature of the study and providing their informed consent to participation
4. Patients willing and able to attend the follow-up visits and procedures foreseen by study protocol.
5. Body mass index (BMI) ≤ 40 kg/m2;
6. Ability to provide written informed consent and can understand and comply with the requirements of the study.

5.2 Exclusion criteria

1. Patients with known inflammatory systemic diseases at the time of enrolment.
2. Patients who are not allowed to undergo the study procedures involving imaging (X-rays, MRI) based on Investigator's judgement.
3. A history of local anaesthetic and anticoagulant drug allergy;
4. Clinically observed active infection in the index knee joint or skin disease/breakdown or infection in the area of the planned injection site of the index knee;
5. Major surgery (e.g. osteotomy) of the index knee within 12 months prior to screening;
6. Minor surgery (e.g. shaving or arthroscopy) of the index knee within 6 months prior to screening;
7. Patients who received intra-articular injection of corticosteroids, PRP or HA within the previous 3 months;
8. Use of systemic immunosuppressants within 6 weeks prior to screening;
9. Patients with a history of invasive malignancies (except non-melanoma skin cancer), unless treated with curative intent and with no clinical signs or symptoms of the malignancy for 5 years;
10. Patients who are participating or have participated in other clinical studies within the 30 days before the study enrolment.
11. Women who are pregnant or breast-feeding or who wish to become pregnant during the period of the clinical investigation and for three months later.

6 Study Design

6.1 General design

This is a post-market clinical investigation of the bone marrow aspirate concentrate (BMAC) and adipose tissue enriched in SVF (at-SVF) for the treatment of OA.

The study is a multicentric, open-label, prospective, 4-arms, in male and female Patients, aged between 18 and 75 years, affected by knee OA Kellgren-Lawrence (KL) 2-3.

The primary objective of this study is to develop an algorithm for the determination of the best treatment for each OA patient, the secondary objective is to identify responder patients to each treatment and to correlate their biologic profile, based on protein expression patterns in the blood samples pre and post-treatment, and the third objective is to compare two autologous bone marrow-

derived and adipose-derived treatments on different OA phenotype patients. High-throughput proteomic analysis will be performed on the patients' sera from the 10 top and 10 worst clinical performers in each group at 6 months. Differentially expressed biomarkers will be validated on the whole cohort of patients by commercially available serum tests (ELISA). Data derived from the clinical trial and biomarker assessment will identify all possible covariates influencing the clinical outcome. A principal components analysis will be the base for the development of a prediction algorithm for identification of responders and non-responders to each treatment, in order to provide indications for a personalized approach for knee OA treatment. The markers identified during the high-throughput analysis will be validated using standard diagnostic procedures. A specific diagnostic kit made of reagents and methods for the assessment of the selected and validated biomarkers will be produced and patented. The proteomics analysis on serum samples will be performed at IRCCS Istituto Ortopedico Galeazzi, while markers validation assays will be performed at IRCCS Humanitas.

240 patients affected by knee OA KL 2-3 will enrolled and randomized in 4 treatment groups:
1) intra-articular injection of BMAC;
2) intra-osseous and intra-articular injection of BMAC;
3) intra-articular injection of at- SVF;
4) intraosseous and intra-articular injection of at-SVF.
Medical history, clinical and radiological features as well as a serum samples, will be collected for each patient at baseline. Complete cell count will be performed on all injected samples.

Patients will be evaluated at the investigational site for 12 months: at Visit 1 (screening visit, day -90 before treatment), Visit 2 (day of treatment), Visit 3 (Follow-up +3 Months after treatment), Visit 4 (Follow-up +6 Months after treatment), Visit 5 (Follow-up +12 Months after treatment).
Adverse events should be assessed and documented at each scheduled visit starting from signature of informed consent. Adverse events will be evaluated at every visit, with an evaluation of pain and the measurement of health status (quality of life and functional scores); radiological assessments (MRI) will be performed at visit 1, visit 4 and visit 5.

6.2 ***Study methods***

Inclusion visit (V1, Day -90)

Informed consent
At the screening visit, the investigator will inform the patient about the study and all the trial procedures.
Patient will be given the information sheet to read and will have time to ask questions on the study. Patient will decide freely whether to participate or not. If he decides to participate, he will be asked to sign the ICF.
The original signed ICF will be retained in the investigator site file and a copy in original will be provided to the participant. Individuals will be free to decline further participation without giving reasons.

Eligibility assessment
The investigator will ensure that each participant meets all the inclusion and exclusion criteria. During this visit that should be performed within 90 days prior baseline visit, the investigator will:
1. Assign Patient Number after informed consent signature
2. Collect patient's demographic characteristics, weight and height
3. Perform physical examination
4. Record Medical history
5. Record prior and concomitant treatments and medications
6. Administer a urine pregnancy test to females of childbearing potential
7. Perform MRI
10. Administer to the patient the following questionnaires:
a. KOOS subscales
b. IKDC questionnaire
c. VAS score
11. Determine eligibility according to inclusion/exclusion criteria
12. Record any AE starting from the informed consent signature

In case of bilateral symptomatic knees, it is recommended that the most symptomatic knee be chosen for treatment in the study, as long as the inclusion/exclusion criteria are met. The knee that does not enter the study can be treated according to general practice.
If during Visit 2 (Baseline Visit/Procedure Visit (arthroscopy), the knee chosen is found to not meet the inclusion/exclusion criteria, the patient will be determined to be intra-operatively ineligible and not be included in this study for both knees.

Treatment Allocation Assessment
Once completed the screening assessments, if found eligible, the subject will be allocated into one of the 4 treatment groups.
The assignment of the patients to different treatment groups will be based on randomization by minimization method, in order to obtain four groups matched for radiological evidence of bone edema, gender and OA grade:
Group A: 1) intra-articular injection of BMAC
Group B: 2) intra-osseous and intra-articular injection of BMAC
Group C: 3) intra-articular injection of adipose-derived SVF
Group D: 4) intraosseous and intra-articular injection of SVF
The enrolment will go on until 60 patients are allocated to each group (allocation:1:1:1:1).

Baseline visit/Procedure Visit (V2, Day 0)
During this visit, the investigator will:
1. Review of inclusion/exclusion criteria and confirm patient's eligibility
2. Administer a urine pregnancy test to females of childbearing potential
3. Peripheral blood samples
4. Harvesting Adipose/Bone Tissue preparation with at-SVF/BMAC Kit

5. Injection can be executed under arthroscopical control
6. Record patient's concomitant treatments and medications
7. Record any AEs
8. Cell count of injected products

Follow-up Visit (V3, 3 months ± 4 weeks)
During this visit, the investigator will:
1. Perform physical examination
2. Perform the knee assessment and complete:
3. Administer to the patient the following questionnaires:
4. KOOS subscales
5. IKDC questionnaire
6. VAS score
7. Record patient's concomitant treatments and medications
8. Record any AEs

Follow-up Visit (V4, 6 months ± 6 weeks)
During this visit, the investigator will:
1. Perform physical examination
2. Perform the knee assessment and complete:
3. Administer to the patient the following questionnaires:
4. KOOS subscales
5. IKDC questionnaire
6. Vas Score
7. Administer a urine pregnancy test to females of childbearing potential, before the MR Imaging
8. Submit patient to a MRI
9. Record patient's concomitant treatments and medications
10. Record any AEs

Follow-up Visit (V4, 12 months ± 8 weeks)
During this visit, the investigator will:
1. Perform physical examination
2. Perform the knee assessment and complete:
3. Administer to the patient the following questionnaires:
4. KOOS subscales
5. IKDC questionnaire
6. Vas Score
7. Administer a urine pregnancy test to females of childbearing potential, before the MR Imaging
8. Submit patient to a MRI
9. Record patient's concomitant treatments and medications
10. Record any AEs

In case of premature withdrawal from the study for whatever reason, the same assessments described for Visit 4 will be performed and recorded in an "Early termination visit".
The Investigator will duly record the reason for premature withdrawal in the source documents and then in the appropriate section of the CRF. Visit 4 (12 months ± 8 weeks) or the 'Early termination Visit' will represent the conclusion of patient's participation in the study.

MRI protocol
MRI for patients will include implementing the Siemens GoKnee3D protocol on a 1.5T Siemens Aera machine.
This will allow us to assess and analyse the morphology of the knee in future finite elements

6.3 Study procedures

General information
In the context of the present clinical investigation the following kits will be used: Hy-tissue SVF (SVAS BIOSANA S.p.A.) and Hy-tissue BMC (Fidia Farmaceutici S.p.A).
Hy-Tissue SVF is an assembled CE-marked system of single-use medical devices for surgical procedures. In the context of the present study the kit will be used for the isolation of the adipose tissue enriched in SVF for autologous transplantation.
Hy-Tissue BMC is an assembled CE-marked system of single-use medical devices for surgical use. In the context of the present study the kit will be used for the isolation of bone marrow concentrate (BMC) for autologous tissue transplantation.

Device supply

The Hy-tissue SVF kit and the Hy-tissue BMC kit will be supplied free of charge by Fidia Farmaceutici S.p.A.

6.4 Treatment modality

6.4.1 Preparation of adipose tissue enriched in stromal vascular fraction

The Hy-tissue SVF kit has to be opened and prepared under sterile conditions following the Manufacturer's instruction. A detailed description of the procedure for the isolation and manipulation of the adipose tissue to obtain the study product will be described in the Investigator's Manual.

6.4.2 Preparation of bone marrow concentrate

The Hy-tissue BMC kit has to be opened and prepared under sterile conditions following the Manufacturer's instruction. A detailed description of the procedure for the isolation and manipulation of the bone marrow to obtain the study product will be described in the Investigator's Manual.

6.5 Proteomic analysis, markers validation and prediction algorithm development

Serum samples will be analyzed by shotgun proteomics after injection into a reverse-phase C18 column in nano-flow UHPLC Easy 1000 coupled with high-resolution mass spectrometer Orbitrap Fusion Tribrid. The top biomarkers significantly different between response-based groups will be chosen based on the magnitude on the observed differences and their biological role in OA, and validated by ELISA in all the study patients. Anamnestic data (age, gender, BMI, habits, co-morbidities, pharmacological treatments, cell content of the injected product), radiological data (MRI) and validated biomarkers will be used to obtain the training dataset for responders and non-responders to each treatment group for the development of a dedicated algorithm. Eventually, based on these results, a diagnostic kit will be developed including specific ELISA tests and identified thresholds.

7 Statistical considerations

7.1 Sample size

Sample Size Calculation: 60 patients will be enrolled in each study group, including a 15% dropout rate. This will allow to record a significant difference of 10 points in KOOS scale among the groups (α=0.05, β=0.1, expected standard deviation=15, t test for independent variables). Moreover, this sample size will allow for the observation of at least 10 non-responders per group with 95% probability, considering a 0.25 frequency in the population. Responder and non-responder patients will be defined on the base of KOOS improvement (difference between KOOS at 6 months and baseline >10 or <10, respectively).

7.2 Analysis

The analysis will be performed using Stata software v 15 (StataCorp LLC). Differences in the proportion of categorical variables among groups will be evaluated by Chi-squared test. Distribution of continuous variables will be assessed by Shapiro-Wilk test and the analysis of possible differences among groups will be performed accordingly. Differences between baseline and 6-month evaluations within groups will be tested by paired t-test or Wilcoxon matched paired test. Further analysis will be conducted using mixed model repeated measures (MMRM) with an unstructured covariance and containing baseline KOOS together with treatment group. The model will be used to estimate the key changes from baseline. The contribution of all relevant covariates (such as age, gender, KL grade, presence of bone oedema, biochemical markers) will be evaluated by multiple regression models, considering also relevant interaction. P-values <0.05 will be considered statistically significant.
Proteomics data will be evaluated using MaxQuant-Andromeda software suite and differences in biomarkers levels will be analysed by Perseus software.

8 Withdrawal of subjects

Screening Failures are defined as participants who are screened and who do not meet the inclusion and exclusion criteria.

A minimal set of screening failure information is required to ensure transparent reporting of screening failure participants.
A patient cannot be enrolled when he does not have all the inclusion criteria and / or has one or more exclusion criteria.

9 Forms and procedures for collecting data and data managing

9.1 Electronic Data Capture Methods

Electronic CRF is used to record study data and it is an integral part of the study and subsequent reports. Therefore, the e-CRF must be completed for each Patient included in the study. Each Patient will be given a specific Patient number. Patient data will be recorded in the e-CRF using this number and will not be known in any other way to any person other than the parties involved in conducting and regulating the study.

9.2 Protocol Deviations

Deviations from the study procedures described in the approved study protocol are not allowed if the deviation affects subject's rights, safety and wellbeing, or the scientific integrity of the clinical investigation. In all this cases a request for deviations need to be submitted in advance to the EC for approval.
Under emergency circumstances, deviations from the study procedures described in the approved study protocol to protect the rights, safety and well-being of human subjects may proceed without prior approval of the EC. Such deviations shall be documented and reported to the EC as soon as possible.

10 Adverse events

The Investigator and any designees are responsible for detecting, documenting, and reporting events that meet the definition of an Adevrse Event (AE) or Serious Adverse Event (SAE) and remain responsible for following-up every AE until the end of the study.
Any device deficiency that could have led to a serious adverse event will also be documented if appropriate action had not been taken, had the intervention not occurred or the circumstances had been less fortunate.
All SAE must be fully recorded and immediately notified (within 24 hours) to the Ivestigator or its designee.
Information regarding serious adverse events will be transmitted to the Investigator-Sponsor or his delegate using the Serious Adverse Event Form, which must be signed by a member of the investigational staff, and to Fidia Safety Surveillance Unit (SSU). The initial report of a serious adverse event may be reported by fax or by telephone. It is preferable that serious adverse events be reported via fax. Subsequent to a telephone report of a serious adverse event, a Serious Adverse Event Form must be completed by the investigational staff and transmitted to the Investigator-Sponsor within 1 working day.

10.1 Adverse Events Definition

An adverse event (AE) is defined as any untoward medical occurrence, unintended disease or injury or any untoward clinical signs (including an abnormal laboratory finding) in subjects, users or other persons whether or not related to the investigational medical device.

Please note:

- This definition includes events related to the investigational device.
- This definition includes events related to the procedures involved.
- For users or other persons, this definition is restricted to events related to investigational medical devices.

10.2 Serious Adverse Event (SAE)

A Serious Adverse Event (SAE) is defined as an AE that results in any of the following:

a) led to a death;

b) led to a serious deterioration in health of the subject, that either resulted in:

- a life-threatening illness or injury, or
- a permanent impairment of a body structure or a body function, or
- in-Patient hospitalization or prolongation of existing hospitalization, or
- in medical or surgical intervention to prevent life threatening illness or injury or permanent impairment to a body structure or a body function

c) led to fetal distress, fetal death or a congenital abnormality or birth defect

Planned hospitalization for pre-existing condition, or a procedure required by the study protocol, without a serious deterioration in health, is not considered a serious adverse event.

10.3 Investigational Medical Device Deficiency

Any malfunction or deterioration in the characteristics and/or performance of a device, as well as any inadequacy in the labeling or the instructions for use which, directly or indirectly, might lead to or might have led to the death of a patient, or USER or of other persons or to a serious deterioration in their state of health.

10.4 Adverse Device Effect (ADE)

Adverse event that are related to the use of an Investigational Medical Device.

An Adverse Device Event (AE) is defined as any untoward medical occurrence, unintended disease or injury or any untoward clinical signs (including an abnormal laboratory finding), related to the Investigational Medical Device.

This includes any adverse event resulting from insufficiencies or inadequacies in the instructions for use, the deployment, the implantation, the installation, the operation, or any malfunction of the investigational medical device.

This includes any event that is a result of a use error or intentional abnormal use of the Investigational Medical Device.

10.5 Serious Adverse Device Effect (SADE)

Adverse device effect that has resulted in any of the consequences characteristic of a SAE.

Anticipated SADE (ASADE): an effect which by its nature, incidence, severity or outcome has been previously identified in the risk analysis report.

10.6 Unanticipated Serious Adverse Device Effect (USADE)

Serious adverse device effect which by its nature, incidence, severity or outcome has not been identified in the current version of the risk analysis report.

10.7 Assessment of Severity (Intensity)

The Investigator will make an assessment of intensity for each AE and SAE reported during the study and assign it to one of the following categories:

- Mild: an event that is easily tolerated by the participant, causing minimal discomfort and not interfering with everyday activities.
- Moderate: an event that causes sufficiently discomfort and interferes with normal everyday activities.
- Severe: an event that prevents normal everyday activities. An AE that is assessed as severe should not be confused with an SAE. Severe is a category utilized for rating the intensity of an event; and both AE and SAE can be assessed as severe.

Please note that an event is defined as “serious” when it meets at least 1 of the predefined outcomes as described in the definition of an SAE (see Section 13.2 - Serious Adverse Event) and NOT when it is rated as “severe”.

10.8 Causality Assessment

The relationship of an AE to the Investigational Medical Device (including the medical surgical procedure) shall be assessed and categorized by the Investigators using the following criteria and definitions.

During causality assessment activity, clinical judgement shall be used and the relevant documents, such as the Investigator’s Brochure, the study protocol or the Risk Analysis Report shall be consulted, as all the foreseeable serious adverse events and the potential risks are listed and assessed there. The presence of confounding factors, such as concomitant medication/treatment, the natural history of the underlying disease, other concurrent illness or risk factors shall also be considered.

Not related	Relationship to the investigational medical device or procedures can be excluded when: - the event is not a known* side effect of the product category the device belongs to or of similar devices and procedures; ** When* the *event is not a known side effect of the product category the device belongs to or of similar devices and procedures, generally is considered "not related". Yet, the unexpected effect shall not be excluded from evaluation and reporting.* - the event has no temporal relationship with the use of the investigational medical device or the procedures; - the serious event does not follow a known response pattern to the medical device (if the response pattern is previously known) and is biologically implausible; - the discontinuation of medical device application or the reduction of the level of activation/exposure - when clinically feasible - and reintroduction of its use (or increase of the level of activation/exposure), do not impact on the serious event; - the event involves a body-site or an organ not expected to be affected by the device or procedure; - the serious event can be attributed to another cause (e.g. an underlying or concurrent illness/clinical condition, an effect of another device, drug, treatment or other risk factors); - harms to the subject are not clearly due to use error; In order to establish the non-relatedness, not all the criteria listed above might be met at the same time, depending on the type of device/procedures and the serious event.
Unlikely	The relationship with the use of the investigational medical device seems not relevant and/or the event can be reasonably explained by another cause, but additional information may be obtained.
Possible	The relationship with the use of the investigational medical device is weak but cannot be ruled out completely. Alternative causes are also possible (e.g. an underlying or concurrent illness/clinical condition or/and an effect of another device, drug or treatment). Cases were relatedness cannot be assessed or no information has been obtained should also be classified as possible.

Probable	The relationship with the use of the investigational medical device seems relevant and/or the event cannot reasonably explained by another cause, but additional information may be obtained.
Causal relationship (Certain)	The serious event is associated with the investigational medical device or with procedures beyond reasonable doubt when: - the event is a known side effect of the product category the investigational medical device belongs to or of similar devices and procedures; - the event has a temporal relationship with investigational medical device use/application or procedures; - the event involves a body-site or organ that: • the Investigational Device or procedures are applied to; • the Investigational Device or procedures have an effect on; - the serious event follows a known response pattern to the investigational medical device (if the response pattern is previously known); - the discontinuation of investigational medical device application (or reduction of the level of activation/exposure) and reintroduction of its use (or increase of the level of activation/exposure), impact on the serious event (when clinically feasible); - other possible causes (e.g. an underlying or concurrent illness/ clinical condition or/and an effect of another device, drug or treatment) have been adequately ruled out; - harm to the subject is due to error in use; - the event depends on a false result given by the investigational medical device used for diagnosis 17, when applicable; In order to establish the relatedness, not all the criteria listed above might be met at the same time, depending on the type of device/procedures and the serious event.

The Investigators will distinguish between the SAEs related to the investigational medical device and those related to the procedures (any procedure specific to the clinical investigation). An AE can be related both to procedures and the investigational medical device. Complications of procedures are considered not related if the said procedures would have been applied to the Patients also in the absence of investigational medical device use/application.
In some particular cases the event may be not adequately assessed because information is insufficient or contradictory and/or the data cannot be verified or supplemented. The Investigators will make the maximum effort to define and categorize the event and avoid these situations. Where the Investigator remains uncertain about classifying the serious event, it should not exclude the relatedness and classify the event as “possible”.
Particular attention shall be given to the causality evaluation of Unanticipated Serious Adverse Device Effects (USADE). The occurrence of such kind of events related to the use of the

investigational medical device could suggest that the clinical investigation places subjects at increased risk of harm than was to be expected beforehand.

11 Ethical considerations

11.1 Patient protection

The responsible investigator will ensure that this study is conducted in agreement with either the Declaration of Helsinki (Tokyo, Venice, Hong Kong and Somerset West amendments) or the laws and regulations of the country.
The protocol has been written, and the study will be conducted according to the ICH Guideline for Good Clinical Practice.
The protocol and its annexes are subject to review and approval by the competent Independent Ethics Committee(s) ("IEC").

11.2 Subject identification – Personal Data protection

All records identifying the subject must be kept confidential and, to the extent permitted by the applicable laws and/or regulations, not be made publicly available. The name of the patient will not be asked for nor recorded at the Data Center. A sequential identification number will be automatically attributed to each patient registered in the study. This number will identify the patient and must be included on all case report forms. In order to avoid identification errors, patient initials and date of birth will also be reported on the case report forms.
Any and all patient information or documentation pertaining to a clinical trial, to the extent permitting, through a "key" kept anywhere, regardless of whether such key is supplied along with the information or documentation or not, must be considered as containing sensitive personal data of the patient, and is therefore subjected to the provisions of applicable data protection ("privacy") regulations. Breach of such regulations may result in administrative or even criminal sanctions.
Particularly, an information sheet prepared according to such regulations and a form to evidence the consent of patients to the processing of such data must therefore accompany the informed consent administered to the patient (see paragraph 14.3 below). Such information must (i) identify the roles of the holder ("titolare") and processor ("responsabile", appointed by the holder) of the patient personal data (also if not directly identifying the patient), as well as the purposes of the personal data collection and processing (medical treatment and related/unrelated scientific research), (ii) adequately describe the flows of communication involving them, particularly if third parties should become involved, and (iii) seek the patient's prior and specific consent to such processing.
Patient information or documentation may be considered "anonymous", and as such not subject to privacy regulations, only when no key whatsoever, permitting the identification of the patient, is any longer available.

11.3 Informed consent

All patients will be informed of the aims of the study. They will be informed as to the strict confidentiality of their patient data, but that their medical records may be reviewed for study purposes by authorized individuals other than their treating physician.

It will be emphasized that the participation is voluntary and that the patient is allowed to refuse further participation in the protocol whenever he/she wants. This will not prejudice the patient's subsequent care. Documented informed consent must be obtained for all patients included in the study before they are registered at the Data Center. This must be done in accordance with the national and local regulatory requirements.
For European Union member states, the informed consent procedure must conform to the ICH guidelines on Good Clinical Practice. This implies that "the written informed consent form should be signed and personally dated by the patient or by the patient's legally acceptable representative".
A copy of Informed Consent should be attached to this Protocol Template.

12 Conflict of interest

Any investigator and/or research staff member who has a conflict of interest with this study (such as patent ownership, royalties, or financial gain greater than the minimum allowable by their institution) must fully disclose the nature of the conflict of interest.

13 Data ownership

The data ownership is regulated by the consortium Agreement signed by the parties.

14 Publication policy

After completion of the study, the project coordinator will prepare a draft manuscript containing final results of the study on the basis of the statistical analysis. The manuscript will be derived to the co-authors for comments and after revision will be sent to a major scientific journal.
All publications, abstracts, presentations, manuscripts and slides including data from the present study will be submitted to and reviewed by the Study Coordinator for coordination and homogeneity purposes: specific advance periods for submission and review may be specified in the protocol. The timing of publications (in the event several Centers should be participating in the Study) may be coordinated, and publication delayed if patentable inventions should be involved (for the time required in order to file the relevant patent applications); otherwise, according to the MoH's Decree of May 12, 2006, investigators cannot be precluded from or limited in publishing the results of their studies (IECs must verify that no excessive restriction is contained in the protocols submitted to their review and approval).

15 Study time table

	Visit 1 Screening/ inclusion visit		**Visit 2** Baseline Visit Procedure	**Visit 3**	Visit 4	Visit 5
	within 90 days prior Visit 2	Allocation	Day 0	3 month	6 month	12 month
Interval Windows				± 4 weeks	± 6 weeks	± 8 weeks
Signed ICF	X					
Eligibility	X	X	X			
Demographics						
Weight, Height, BMI	X					
Physical Examination	X			X	X	X
Medical History	X					
Urine pregnancy test	X				X	X
Prior and Concomitant treatments and medications	X			X	X	X
Questionnaires	X			X	X	X
MRI	X				X	X
Allocation to treatment: Group A: 1) intra-articular injection of BMAC		X*				

Group B: 2) intra-osseous and intra-articular injection of BMAC Group C: 3) intra-articular injection of adipose-derived SVF Group D: 4) intraosseous and intra-articular injection of SVF						
Surgical Operation			X			
Peripheral blood samples			X			
Adverse Events	X	X	X	X	X	X

*Randomization by minimization method will be performed to obtain four groups matched for radiological evidence of bone edema, age, gender and OA grade.

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