PROTOCOL

MIcrobiota as earLy diagnostic and predictivE factor for oSTeOarthritic degeNEration and microbial contamination (MILESTONE)

Protocol Code: MILESTONE

Version No. (Date): V2.0 (13/01/2025)

Sponsor (Institution): IRCCS Istituto Clinico Humanitas

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COORDINATING INVESTIGATOR

I have approved this Protocol entitled "MIcrobiota as earLy diagnostic and predictivE factor for oSTeOarthritic degeNEration and microbial contamination (MILESTONE)" and I agree to conduct the study as detailed herein and according to the current version of the World Medical Association Declaration of Helsinki, Good Clinical Practice guideline and applicable regulatory requirements. I will provide all study personnel under my supervision with all information needed to perform the study and I will inform them about their responsibilities and obligations

Printed name	Tommaso Bonanzinga
Role	Principal Investigator
Department	Center for the functional and biological reconstruction of the knee joint
Signature	
Date	13/01/2025

STATISTICIAN

I have approved this Protocol entitled "MIcrobiota as earLy diagnostic and predictivE factor for oSTeOarthritic degeNEration and microbial contamination (MILESTONE)"

Printed name	Emanuela Morenghi
Signature	
Date	13/01/2025

CENTRE SIGNATURE – PRINCIPAL INVESTIGATOR

I have read this Protocol Amendment relevant to the study entitled "MIcrobiota as earLy diagnostic and predictivE factor for oSTeOarthritic degeNEration and microbial contamination (MILESTONE)" 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 Coordinating Investigator/Sponsor and I will inform them about their responsibilities and obligations.

Printed name	Tommaso Bonanzinga
Role	Principal Investigator
Department	Center for the functional and biological reconstruction of the knee joint
Signature	
Date	13/01/2025

Abbreviations

CA	Competent Authority
EC	Ethics Committee
eCRF	electronic Case report form
GCP	Good Clinical Practice
IMP	Investigational Medicinal Product

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1. SUMMARY

Study Title	MIcrobiota as earLy diagnostic and predictivE factor for oSTeOarthritic degeNEration and microbial. Contamination		
Study code		**Acronym:**	MILESTONE
Version and Date	V 2.0 13/01/2025		
Sponsor (Institution)	IRCCS Istituto Clinico Humanitas		
Coordinating Investigator	Tommaso Bonanzinga		
Supporter	NA		
Product Name	NA		
Study indication	Osteoarthritis		
Background and rationale	Recent studies have highlighted the role of gut microbiota in the pathogenesis of a variety of human metabolic, immunological, and neurological diseases such as inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS), cardiovascular disease, and certain types of cancer [1]. In the field of orthopedics, the gut microbiota has gained attention as an environmental factor that can influence the development and progression of osteoarthritis (OA) [2]. It is now understood that OA is not solely a disease of mechanical wear and tear but involves complex interactions between genetic, environmental, and lifestyle factors [3]. The gut microbiota has gained attention as one of the environmental factors that can influence the development and progression of OA. It has been postulated that gut dysbiosis could exert its influence on the joint by establishing a chronic low-grade inflammation, altering the production of fatty acids which influences cartilage and modulation of pain perception [4]. Pre-clinical studies on animals have indeed confirmed this link but no study on humans has yet been published in the literature. [5] The goal of this study is to investigate how gut microbiota could influence the correct functioning of the body's main joint and how it affects the development of early OA, Periprosthetic Joint Infection (PJI), and recovery after total joint replacement. We hypothesized that gut microbiota could influence the correct functioning of the main joint of the body, and therefore affect the development of early OA, of PJI and even the recovery after total joint replacement. If this hypothesis was confirmed by the results of the current study, it would change our understanding of osteoarthritis and periprosthetic joint infections providing a novel target for our preventive and possibly even therapeutic strategies. For example, probiotics, prebiotics, postbiotics and fecal microbiota transplantation		

	(FMT) could become new weapons in the orthopedic field. If gut dysbiosis will be found to be a predictive factor of early osteoarthritis, this will allow early recognition of the condition and therefore a precocious management of the condition [6]. In addition, the discovery that microbiota influences recovery after joint replacement could help us improving the post-operative care and ameliorating the recovery. This would translate in an improvement of the quality of life for the patient and a reduction of the costs for the national healthcare system. [1] Gebrayel P. et al. Microbiota medicine: towards clinical revolution. J Transl Med 2022 [2] Hao X. et al. The gut microbiota in osteoarthritis: where do we stand and what can we do? Arthritis Res Ther 2021 [3] Martel-Pelletier J. et al. NRDP 2016 [4] Hao X. et al. AR Ther 2021 [5] Tan TC et al. IJRD 2021 [6] Chu, C.R., Williams, A.A., Coyle, C.H. et al. Early diagnosis to enable early treatment of pre-osteoarthritis. Arthritis Res Ther 14, 212 (2012).
Study Objectives	Objective #1: to estimate the prevalence of early OA among patients with gut dysbiosis. Objective #2: to investigate the intra-articular microbiota during surgery; fecal and oral microbiota, before surgery and during recovery; permeability markers from blood and feces before surgery. Objective #3: to describe gut microbiota environment predisposing to gut dysbiosis, which negatively affects life expectancy in these patients, in periprosthetic joint infections (PJI) patients.
Study design	Multicentric observational
Eligibility Criteria	<u>For objective #1 (to estimate the prevalence of early OA among patients with gut dysbiosis)</u> Inclusion criteria: gut dysbiosis; age range 18-50 years old Exclusion criteria: musculoskeletal symptoms; previous shoulder or knee surgery; previous shoulder or knee known pathological conditions; rheumatological diseases <u>For objective #2 (to investigate the intra-articular microbiota during surgery; fecal and oral microbiota, before surgery and during recovery; permeability markers from blood and feces before surgery)</u> Inclusion criteria: patients undergoing total knee replacement surgery Exclusion criteria: any concurrent or previous diseases or conditions which might negatively affect the surgery <u>For objective #3 (to describe gut microbiota environment predisposing to gut dysbiosis, which negatively affects life expectancy in these patients, in periprosthetic joint infections (PJI) patients)</u>

	Inclusion criteria: patients undergoing joint revision surgery for septic failure of a knee or hip prostheses or patients undergoing revision surgery for aseptic mobilization for PJI Exclusion criteria: major predisposing factor for gut dysbiosis (antibiotic therapy in the last 6 months, BMI > 40 and inflammatory bowel disease); chronic inflammatory joint diseases (e.g., rheumatoid arthritis, psoriatic arthritis); acute (< 90 days after the index procedure) and late hematogenous (symptoms of less than three weeks duration) infections; an inadequate amount of synovial fluid (< 10 mL) for culture, WBC, and PMN (neutrophil) percentage determinations
Study Procedures	The study is a multicentre observational study divided into 3 objectives: Objective #1 is to enrol 40 patients with established intestinal dysbiosis and no musculoskeletal symptoms. Patients with potential dysbiosis will undergo an initial (V1) gastroenterological examination at the Operative Unit of Gastroenterology and Digestive Endoscopy in Humanitas, where they will be asked to join the clinical study. Each patient will be explained the study through the means of the informed consent process, and those who wish to join the study will sign the informed consent form. For each patient, the Bristol scale will be used as a tool to categorize the patients' stool as reported by the patient; a stool sample collection kit will be provided to the patient together with its instructions for use. Patients will be instructed to provide a stool sample (V2) at the immunology and microbiota unit laboratory. On the same occasion, the clinician will perform a buccal swab to collect a saliva sample from the patient (see section 8 for specifics). Both samples will initially be stored at the Biobank - Cancer Center (Istituto Clinico Humanitas - Via Manzoni 56, 20089 Rozzano (MI)) under the responsibility of Dr. Daniela Pistillo and will then be processed at the laboratory of the immunology and microbiota unit and will be used for confirming the dysbiosis diagnosis. If the patient is not positive for dysbiosis markers, he/she will be classified as SCREENING FAILURE; if the dysbiosis is confirmed, the patient will be asked to come to another visit (V3) where he/she will undergo a MRI of the knee and a shoulder ultrasound scan in order to check for early osteoarthritis signs. Optional orthopaedic examination may be performed. Blood test will also be performed part of the samples of which will be used to evaluate biomarkers and will initially be stored at the Biobank - Cancer Center (Istituto Clinico Humanitas - Via Manzoni 56, 20089 Rozzano (MI)) with Dr. Daniela Pistillo in charge and will subsequently be processed. The other part of the blood samples on which insulin, glycated haemoglobin and glucose will be analysed will be sent to the Central Analysis Laboratory within ICH (see section 8 for details). Objective #2 is to enrol 50 patients undergoing total knee arthroplasty (TKA). Patients scheduled to undergo TKA will undergo a pre-hospitalization orthopaedic examination (V1) where he/she will be informed about the clinical study. If he/she agrees to join the study,

	he/she will sign the informed consent form and will follow study procedures: questionnaires will be administered and the patient will receive a stool sample collection kit together with its instructions for use. The stool sample will be used for DNA extraction at the immunology and microbiota unit laboratory and subsequent metagenomic analysis. Patient will be instructed to hand over the stool sample during hospital visit for TKA (V2), during which intra-articular tissue samples will be collected for metagenomics. In addition, buccal swab and blood samples of the patient undergoing the surgery will be collected before surgery. Saliva, stool and blood samples for biomarkers will initially be stored at the Biobank - Cancer Center (Istituto Clinico Humanitas - Via Manzoni 56, 20089 Rozzano (MI)) under the responsibility of Dr. Daniela Pistillo and will subsequently be processed at the laboratory of the immunology and microbiota unit. The other part of the blood samples on which insulin, glycated haemoglobin and glucose will be analysed will be sent to the ICH's in-house Central Analysis Laboratory. On discharge, patient will be provided another stool collection kit. One month after surgery (V3), a follow-up examination will be performed, where patient will bring the second stool sample and will undergo another buccal swab which will also be analysed by the same laboratory (see section 8). For stool and saliva samples of V3, the storage and analysis procedure is the same as for V2. In addition, the patient will fill in the KSS questionnaires. Objective #3 involves the enrolment of 80 patients undergoing joint revision surgery who are further divided into: Group 1: 40 patients for septic failure of a knee or hip replacement. Group 2: 40 patients for aseptic loosening for PJI Patients will undergo a pre-hospitalization visit (V1) with orthopaedic check-up, where they will be asked to join the clinical study. Each patient will be explained the study through the means of the informed consent process, and those who wish to join the study will sign the informed consent form. Patient will be provided a stool sample collection kit for DNA extraction at the immunology and microbiota unit laboratory and subsequent metagenomic analysis. The patient will be instructed to hand over the stool sample during hospital visit for revision surgery (V2) (see section 8).
Number of patients (planned)	Three objectives are set and a total of 170 patients are divided as following: Objective #1: 40 patients with proven gut dysbiosis and without musculoskeletal symptoms undergoing MRI of the knee and the ultrasound of the shoulder. Objective #2: 50 patients undergoing total knee replacement. Objective #3: 80 patients, which are further subdivided into: Group 1: 40 patients undergoing joint revision surgery for septic failure of a knee or hip prostheses. Group 2: 40 patients undergoing revision surgery for aseptic mobilization for PJI.

Investigational Sites (planned)	Single-centre study □ Multi-centre study x 1: Centres: IRCCS Istituto Clinico Humanitas (Rozzano) 2: AORN Antonio Cardarelli (Napoli)
Sample size and statistical consideration	Regarding the different objective of the study, Experimental design objective #1 and Experimental design objective #3, had the primary aim to describe the sample, so the study will enroll all the patients fulfilling inclusion and exclusion criteria, estimable in around 40 for Experimental design objective #1 and 80 for Experimental design objective #3. Regarding Experimental design objective #2, we can expect a difference in KSS between patient with or without microbiota of 9 points. With an alpha error of 0.05 and a power of 0.8, considering a standard deviation of 11, the study will enroll 25 patients with microbiota and 25 without, for a total of 50 patients.
Study timetable	Provide the following study milestones: • Planned date of the First Patient In (FPI - date of the Informed Consent signature of the first study patient): february 2025 • Planned date of the Last Patient In (LPI - date of the Informed Consent signature of the last study patient): february 2026 • Planned date of the Last Patient Out (LPO – date of the last visit of the last study patient): february 2026 • Planned study duration (from FPI to LPO): 24 months
GCP Statement:	This study will be conducted in compliance with the protocol, the current version of the Declaration of Helsinki and applicable guidelines as well as all national legal and regulatory requirements.

2. STUDY FLOW-CHART

Objective #1

Assessment / events	V1	V2	V3
ICF signed	x		
Demographic data	x		
Medical history	x		
Gastroenterological examination	x		
Kit for stool sample collection handover	x		
Stool sample collection		x	
Saliva sample collection		x	
Blood analysis			x
Magnetic resonance imaging of the knee			x
Ultrasound of the shoulder			x
Orthopedic examination			x*

*: optional

Objective #2

Assessment / events	V1	V2	V3 (1 M after surgery)
ICF signed	x		
Demographic data	x		
Medical history	x		
Kit for stool sample collection handover	x	x	
Stool sample collection		x	x
Blood analysis		x	
Intra-articular sampling		x	
Total knee replacement		x	
Saliva sample		x	x
KSS score	x		x
Orthopaedic visit	x		x

Objective #3

	V1	V2
Assessment / events	x	

ICF signed	x	
Demographic data	x	
Medical history	x	
Kit for stool sample collection handover	x	
Stool sample collection		x
Reintervention		x

3. BACKGROUND

Recent studies have highlighted the role of gut microbiota in the pathogenesis of a variety of human metabolic, immunological, and neurological diseases such as inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS), cardiovascular disease, and certain types of cancer[1]. In the field of orthopedics, the gut microbiota has gained attention as an environmental factor that can influence the development and progression of osteoarthritis (OA)[2]. It is now understood that OA is not solely a disease of mechanical wear and tear but involves complex interactions between genetic, environmental, and lifestyle factors [3]. The gut microbiota has gained attention as one of the environmental factors that can influence the development and progression of OA. It has been postulated that gut dysbiosis could exert its influence on the joint by establishing a chronic low-grade inflammation, altering the production of fatty acids which influences cartilage and modulation of pain perception [4]. Pre-clinical studies on animals have indeed confirmed this link but no study on humans has yet been published in the literature. [5]

The goal of this study is to investigate how gut microbiota could influence the correct functioning of the body's main joint and how it affects the development of early OA, Periprosthetic Joint Infection (PJI), and recovery after total joint replacement.

[1] Gebrayel P. et al. Microbiota medicine: towards clinical revolution. J Transl Med 2022
[2] Hao X. et al. The gut microbiota in osteoarthritis: where do we stand and what can we do? Arthritis Res Ther 2021
[3] Martel-Pelletier J. et al. NRDP 2016
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[5] Tan TC et al. IJRD 2021
[6] Chu, C.R., Williams, A.A., Coyle, C.H. et al. Early diagnosis to enable early treatment of pre-osteoarthritis . Arthritis Res Ther 14, 212 (2012).

4. RATIONALE

We hypothesized that gut microbiota could influence the correct functioning of the main joint of the body, and therefore affect the development of early OA, of PJI and even the recovery after total joint replacement. If this hypothesis was confirmed by the results of the current study, it would change our understanding of osteoarthritis and periprosthetic joint infections providing a novel target for our preventive and possibly even therapeutic strategies. For example, probiotics, prebiotics, and fecal microbiota transplantation (FMT) could become new weapons in the orthopedic field. If gut dysbiosis will be found to be a predictive factor of early osteoarthritis, this will allow early recognition of the condition and therefore a precocious management of the condition [1]. In addition, the discovery that microbiota influences recovery after joint replacement could help us improving the post-operative care and ameliorating the recovery. This would translate in an improvement of the quality of life for the patient and a reduction of the costs for the national healthcare system.

[1] Chu, C.R., Williams, A.A., Coyle, C.H. et al. Early diagnosis to enable early treatment of pre-osteoarthritis. Arthritis Res Ther 14, 212 (2012).

5. STUDY OBJECTIVES

Experimental design Objective #1: to estimate the prevalence of early OA among patients with gut dysbiosis.
Experimental design Objective #2: to investigate the intra-articular microbiota during surgery; fecal and oral microbiota, before surgery and during recovery; permeability markers from blood and feces before surgery.
Experimental design Objective #3: to describe gut microbiota environment predisposing to gut dysbiosis, which negatively affects life expectancy in these patients, in periprosthetic joint infections (PJI) patients

6. STUDY POPULATION

A total of 170 patients are divided as following across the three study objectives:

Experimental design objective #1: 40 patients with proven gut dysbiosis and without musculoskeletal symptoms undergoing MRI of the knee and the ultrasound of the shoulder.

Experimental design objective #2: 50 patients undergoing total knee replacement.

Experimental design objective #3: 80 patients are further subdivided into:
Group 1: 40 patients undergoing joint revision surgery for septic failure of a knee or hip prostheses.
Group 2: 40 patients undergoing revision surgery for aseptic mobilization for PJI.

7. INCLUSION AND EXCLUSION CRITERIA

7.1. INCLUSION CRITERIA

For objective #1 (to estimate the prevalence of early OA among patients with gut dysbiosis)

Inclusion criteria: proven gut dysbiosis (increased Firmicutes/Bacteroidetes (F/B) phyla ratio and increased permeability); age range 18-50 years old.

For objective #2 to investigate the intra-articular microbiota during surgery; fecal and oral microbiota, before surgery and during recovery; permeability markers from blood and feces before surgery.

Inclusion criteria: patients undergoing total knee replacement surgery

For objective #3 (to describe gut microbiota environment predisposing to gut dysbiosis, which negatively affects life expectancy in these patients, in periprosthetic joint infections (PJI) patients)

Inclusion criteria: patients undergoing joint revision surgery for septic failure of a knee or hip prostheses; patients undergoing revision surgery for aseptic mobilization for PJI

7.2 EXCLUSION CRITERIA

For objective #1 (to estimate the prevalence of early OA among patients with gut dysbiosis)

Exclusion criteria: musculoskeletal symptoms; previous shoulder or knee surgery; previous shoulder or knee known pathological conditions; rheumatological diseases

For objective #2 to investigate the intra-articular microbiota during surgery; fecal and oral microbiota, before surgery and during recovery; permeability markers from blood and feces before surgery.

Exclusion criteria: any concurrent or previous diseases or conditions which might negatively affect the surgery

For objective #3 (to describe gut microbiota environment predisposing to gut dysbiosis, which negatively affects life expectancy in these patients, in periprosthetic joint infections (PJI) patients)
Exclusion criteria: major predisposing factor for gut dysbiosis (antibiotic therapy in the last 6 months, BMI > 40 and inflammatory bowel disease); chronic inflammatory joint diseases (e.g., rheumatoid arthritis, psoriatic arthritis); acute (< 90 days after the index procedure) and late hematogenous (symptoms of less than three weeks duration) infections; an inadequate amount of synovial fluid (< 10 mL) for culture, WBC, and PMN (neutrophil) percentage determinations

7.3. RECRUITMENT

Objective #1: young (18-50 years old) patients with potential dysbiosis who are scheduled to undergo a gastroenterological consultation will be explained the purposes of the clinical study during the consultation

through the means of the informed consent process. Patients will be asked to provide a stool sample and a saliva sample (buccal swab), which will be assessed in order to confirm the dysbiosis. Upon diagnosis confirmation, patients will join the clinical study and will undergo follow up examinations as scheduled in the protocol.

Objective #2: during the course of a routine pre-surgery assessment visit, patients who comply with the inclusion/exclusion criteria who are already scheduled to undergo total knee replacement will be explained the clinical study; if the patients agree to join the study, they will undergo follow up examinations as scheduled in the protocol.

Objective #3: patients who comply with the inclusion/exclusion criteria who present with failed or painful total hip or knee arthroplasty will undergo standardized diagnostic protocol to identify those with a PJI. The diagnosis of chronic infection (> 90 days after the index procedure) will be made based on the International Consensus Group (ICM) definition of PJI. Site staff will explain the clinical study and patients will then be asked to join the clinical study during the pre-surgery assessment visit. If the patients agree to join the study, they will undergo follow up examinations as scheduled in the protocol.

8. STUDY ASSESSMENT

Prior to conducting any of the pre-entry test not performed routinely in the treatment of the patient, the investigator will:

- explain the study to the patient by means of the informed consent process
- collect a signed copy of written informed consent.

The study is a multicentre observational study divided into 3 objectives:

Objective #1 is to enrol 40 patients with established intestinal dysbiosis and no musculoskeletal symptoms. Patients with potential dysbiosis will undergo an initial (V0) gastroenterological examination at the Operative Unit of Gastroenterology and Digestive Endoscopy in Humanitas, where they will be asked to join the clinical study. Each patient will be explained the study through the means of the informed consent process, and those who wish to join the study will sign the informed consent form. For each patient, the Bristol scale will be used as a tool to categorize the patients' stool as reported by the patient; a stool sample collection kit will be provided to the patient together with its instructions for use. Patients will be instructed to provide a stool sample (V1) at the immunology and microbiota unit laboratory. On the same occasion, the clinician will perform a buccal swab to collect a saliva sample from the patient. (see section 8 for specifics). Both samples will initially be stored at the Biobank - Cancer Center (Istituto Clinico Humanitas - Via Manzoni 56, 20089 Rozzano (MI)) under the responsibility of Dr. Daniela Pistillo and will then be processed at the laboratory of the immunology and microbiota unit and will be used for confirming the dysbiosis diagnosis. be used for confirming the dysbiosis diagnosis. If the patient is not positive for dysbiosis markers, he/she will be classified as SCREENING FAILURE; if the dysbiosis is confirmed, the patient will be asked to come to another visit (V3) where he/she will undergo a MRI of the knee and a shoulder ultrasound scan in order to check for early osteoarthritis signs. Optional orthopaedic examination may be performed. Blood test will also be performed part of the samples of which will be used to evaluate biomarkers and will initially be stored at the Biobank - Cancer Center (Istituto Clinico Humanitas - Via Manzoni 56, 20089 Rozzano (MI)) with Dr. Daniela Pistillo in charge and will subsequently be processed. The other part of the blood samples on which insulin, glycated haemoglobin and glucose will be analysed will be sent to the Central Analysis Laboratory within ICH (see section 8 for details).

Objective #2 is to enrol 50 patients undergoing total knee arthroplasty (TKA). Patients scheduled to undergo TKA will undergo a pre-hospitalization orthopaedic examination (V1) where he/she will be informed about the clinical study. If he/she agrees to join the study, he/she will sign the informed consent form and will follow study procedures: questionnaires will be administered and the patient will receive a stool sample collection kit

together with its instructions for use. The stool sample will be used for DNA extraction at the immunology and microbiota unit laboratory and subsequent metagenomic analysis. Patient will be instructed to hand over the stool sample during hospital visit for TKA (V2), during which intra-articular tissue samples will be collected for metagenomics. In addition, buccal swab and blood samples of the patient undergoing the surgery will be collected before surgery. Saliva, stool and blood samples for biomarkers will initially be stored at the Biobank - Cancer Center (Istituto Clinico Humanitas - Via Manzoni 56, 20089 Rozzano (MI)) under the responsibility of Dr. Daniela Pistillo and will subsequently be processed at the laboratory of the immunology and microbiota unit. The other part of the blood samples on which insulin, glycated haemoglobin and glucose will be analysed will be sent to the ICH's in-house Central Analysis Laboratory. On discharge, patient will be provided another stool collection kit. One month after surgery (V3), a follow-up examination will be performed, where patient will bring the second stool sample and will undergo another buccal swab which will also be analysed by the same laboratory (see section 8). For stool and saliva samples of V3, the storage and analysis procedure is the same as for V2. In addition, the patient will fill in the KSS questionnaires.

Objective 3 involves the enrolment of 80 patients undergoing joint revision surgery who are further divided into:

Group 1: 40 patients for septic failure of a knee or hip replacement.

Group 2: 40 patients for aseptic loosening for PJI

Patients will undergo a pre-hospitalization (V1) with orthopaedic check-up, where they will be asked to join the clinical study. Each patient will be explained the study through the means of the informed consent process, and those who wish to join the study will sign the informed consent form. Patient will be provided a stool sample collection kit for DNA extraction at the immunology and microbiota unit laboratory and subsequent metagenomic analysis. The patient will be instructed to hand over the stool sample during hospital visit for revision surgery (V2) (see section 8).

Details for sampling

The following samples will be collected:

1. Feces (microbiota and biomarker of intestinal permeability)
2. Saliva (buccal swab) (microbiota)
3. Intra-articular tissue (microbiota)
4. Peripheral blood (serum collected in EDTA) for the evaluation of biomarkers associated with intestinal permeability biomarkers, fasting glucose and insulin, glycated haemoglobin (HbA1c).

Fecal sampling

The feces will be collected by the patient within 24 hours preceding the hospital visit. Detailed instructions and the container for the collection will be provided to the patient. The container will be transferred to the hospital and then to the laboratories at +4°C. Once in the laboratories the sample will be stored at -80°C.

Oral sampling*:* Saliva samples will be collected using a buccal swab performed by a healthcare professional, and frozen as soon as possible at – 80°C for subsequent microbiome analysis. It is recommended to perform the test when the mouth is clean, as external substances can skew the analysis results. It is advised to collect the sample before taking any oral medications and to avoid smoking, eating, chewing gum, drinking coffee, or any beverages other than water for 30 minutes prior to the collection. It is recommended to drink water 15 minutes before the collection to facilitate salivation. To increase saliva production, the patients should massage the inside of their cheeks with their tongue and to push the tip of their tongue against their teeth. Yawning or making chewing motions can also help increase salivation.

Intra-articular tissue sampling for microbiome analysis: For each patient, 5 intra-articular samples will be taken intraoperatively: two SF (sinovial fluid) samples, one from ST (sinovial tissue), one from cartilage and one from Hoffa's fat pad. They are then analyzed as follows: bacterial DNA will be extracted using DNeasy PowerSoil Pro Kits (Qiagen) according to the manufacturer's procedures. DNA concentration will be measured using the NanoDrop spectrophotometer (Thermo Fisher scientific) and stored at −80 °C. Libraries will be prepared using Illumina® DNA prep according to the manufacturer's procedures. 16S rRNA gene sequencing will be carried out. Standard microbiota diversity metrics (e.g., alpha diversity, including species richness and evenness, beta-diversity, rarefaction curves) and taxonomy will be calculated.

Blood sampling: Prior to surgery, peripheral blood samples will be collected from each patient enrolled in the observational study into 2 BD Vacutainer SST II Advanced (REF 367953) and will be incubated 30' at room T°. Tubes will then be centrifuged 2000 g with maximum acceleration and slowing down (9/9) at Room T° 21°C 15'. In sterile conditions, serum will be drawn with P1000 pipette tip with sterile filter. The serum will be employed for assessing intestinal permeability-associated biomarkers, fasting glucose and insulin, glycated haemoglobin (HbA1c).

Oral, and fecal microbiome analysis: Bacterial DNA will be extracted using DNeasy PowerSoil Pro Kits (Qiagen) according to the manufacturer's procedures. DNA concentration will be measured using the NanoDrop spectrophotometer (Thermo Fisher scientific) and stored at −80 °C. Libraries will be prepared using Illumina® DNA prep according to the manufacturer's procedures. Shotgun metagenomic sequencing will be performed for buccal (~ 15 Gb/sample) and fecal (~ 5-7.5 Gb/sample) samples. Standard microbiota diversity metrics (e.g., alpha diversity, including species richness and evenness, beta-diversity, rarefaction curves) will be calculated.Taxonomic profiling will be performed by using the latest version available of MetaPhlAn and the latest taxonomic database available. Output: MetaPhlAn 4 taxonomy tables with relative abundances of species/SGBs. Functional profiling will be performed by using the latest version available of HUMAnN. Output: HUMAnN tables with gene families UniRef90, RPKMs, BioCyc path RPKMs and path coverage in .tsv files.

Analysis of biomarkers associated with intestinal permeability: Fecal and blood samples will be used to measure, by specific ELISA kits, intestinal permeability biomarkers: e.g Zonulin will be measured both in the serum and in the feces; bacterial DNA, I-FABP and LBP will be measured in the serum.

9. STATISTICAL CONSIDERATIONS

9.1. Sample size

Regarding the different objective of the study, Experimental design objective #1 and Experimental design objective #3, had the primary aim to describe the sample, so the study will enroll all the patients fulfilling inclusion and exclusion criteria, estimable in around 40 for Experimental design objective #1 and 80 for Experimental design objective #3.
Regarding Experimental design objective #2, we can expect a difference in KSS between patient with or without microbiota of 9 points. With an alpha error of 0.05 and a power of 0.8, considering a standard deviation of 11, the study will enroll 25 patients with microbiota and 25 without, for a total of 50 patients.

9.2. Analysis

Data will be described as number and percentage, if categorical, or mean and standard deviation, if continuous, approximately Gaussian, or median and range, otherwise. Adherence to Gaussian distribution will be checked with Shapiro Wilks test.
For Experimental design objective #1 and Experimental design objective #3 only descriptive statistics will be performed. For Experimental design objective #2, differences between the two groups will be explored with

chi square test, for continuous variables, or with t student test or with Mann Whitney test for numerical variables, according to their distribution. Association with arthroplasty recovery will be evaluated with logistic regression analysis, and the results will be expressed as odds ratio (OR), with 95% confidence interval (95%CI).
Significance threshold will be set to 0.05. All analyses will be made with Stata version 18 or superior.

10. QUALITY ASSURANCE AND CONTROL

Quality Assurance and Quality Control systems based on written SOPs are in place at the Sponsor site.

10.1. Data handling and record keeping / archiving

The investigator must keep the documents on file for at least 7 years after completion or discontinuation of the study. After that period, the documents may be destroyed, subject to local regulations. Before proceeding to documents' destruction, sites must inform the Coordinating Investigator/delegate in writing.

Should the investigator wish to assign the study records to another party or move them to another location, the Coordinating Investigator/delegate must be notified in advance.

10.2. Case Report Forms

After having signed the informed consent form (ICF) and privacy form, patients' clinical, demographic and radiological data will be recorded during each visit in the visit report. Any data relevant to the study protocol will be recorded in Excel files which are protected by a password and who will be accessed only by study staff.
The investigator will ensure the accuracy, completeness and timeliness of the data reported in the files. Participants will not be identified in the Excel files by name or initials and birth date. Appropriate coded identification will be used.

10.3. Source documents

Source data must be available at the site to document the existence of the study participants. Source data include the original documents relating to the study, as well as the medical treatment and medical history of the participant.

11. CONFIDENTIALITY OF PATIENT RECORDS

The investigator assures that patients' anonymity should be maintained and that their identities are protected from unauthorized parties. Particular attention should be paid whenever patient data are supplied to third parties and may be autonomously processed.

The investigator should keep in a confidential way a patient identification log recording both patient code and name. The investigator should also maintain patients' written consent forms, in strict confidence (i.e. not for submission to the Coordinating Investigator).

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.

12. ETHICAL CONSIDERATIONS

The responsible investigator ensures that this study is conducted in agreement with this protocol, the Good Clinical Practice, the current version of Declaration of Helsinki and the applicable regulations.

The protocol and any amendments are subject to review and approval by the competent Independent Ethics Committee(s) ("IEC").

13. INFORMED CONSENT

All patients should be informed of the aims of the study. They should 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 should 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 does not prejudice the patient's subsequent care. Documented informed consent must be obtained for all patients included in the study before any study related procedure is performed. The written informed consent form should be signed and personally dated by the patient or by the patient's legally acceptable representative, and by the Investigator who has provided the study information.

14. DATA OWNERSHIP

Istituto Clinico Humanitas is the owner of the data resulting from the study. All centers and investigators participating in the study should be made aware of such circumstance and not to disseminate information or data without the prior written consent by Istituto Clinico Humanitas.

15. PUBLICATION POLICY

After completion of the study, the Coordinating Investigator prepares a draft manuscript containing final results of the study on the basis of the statistical analysis. The manuscript is delivered to the co-authors for comments and then sent to a scientific journal for publication.

All publications, abstracts, presentations, manuscripts and slides - issued by the Investigators of the collaborative sites and including data from the present study- should be submitted to and reviewed by the Coordinator Investigator at least 3 (three) weeks in advance the planned date for the submission to the scientific journal.

16. FUNDING AND SUPPORT

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