**Progetto 3. Diagnostic and therapeutic potential of the long pentraxin PTX3 in bacterial infections of the bone**, che sarà sviluppato nel periodo 2022-2024 presso Humanitas University a cui partecipano ricercatori del National Research Council-Institute for Genetic and Biomedical Research (CNR-IRGB), National Research Council-Institute of Science and Technology for Ceramics (CNR-ISTEC), Faenza (RA), University of Zagreb, Zagreb – Croatia

**Executive summary**

Bone infections, including Staphylococcus aureus (SA)-dependent osteomyelitis (OM) secondary to trauma and arthroplasty, are severe diseases, often characterized by incomplete functional reconstitution and progressive frailty of the affected bone. Over the last three years, we have demonstrated that the long pentraxin PTX3, a soluble protein of the innate immune system at the interface between infection immunity and bone biology, plays a role in OM pathogenesis.
In this regard, we have shown that:
a. in a mouse model of SA-OM, PTX3 aggravates the disease while the absence of this protein is associated with less severe pathology, pointing to the pharmacological blockade of PTX3 as a new strategy in the therapy of bone infections;
b. the concentration of the PTX3 protein in the synovial fluid specifically predict infection in patients undergoing revision arthroplasty, which is of clinical relevance in the management of OM patients.

Building upon these findings and our past experience, we will:
a. characterize the molecular mechanism(s) of PTX3 in the pathogenesis of OM;
b. evaluate the pharmacological blockade of PTX3 and its combination with nano-delivered antibiotics in the prophylaxis/therapy of OM;
c. assess and validate the diagnostic potential of PTX3 (and other selected genes) as a genetic biomarker in periprosthetic joint infections (PJIs).

These objectives will be pursued in a translational research project that integrates in vivo modelling of OM (in a murine model of SA-OM), in vitro experimentation (on bone biomimetic 3D scaffolds), and clinical studies (both in retrospective and prospective cohorts of PJI patients).

**Project proposal**

Osteomyelitis (OM) is an infectious disease of the bone primarily caused by the opportunistic pathogen Staphylococcus aureus (SA). OM can arise from hematogenous spreading of the microbe, or after a trauma (i.e., fracture or surgery), and is associated with intense local inflammation, extensive tissue remodeling (with abnormal periosteal bone formation and bone destruction), and pain. Aggressive antimicrobial therapies are the standard of care, however multiple revision surgeries are often needed that result into incomplete functional reconstitution and progressive frailty of the infected bone. Hence, elevated are the healthcare and social costs of OM, which calls for timely research into its pathogenetic mechanisms to orient and improve patient management.
The bone is a favorable environment for SA adhesion and seeding through microbial recognition of host cell receptors and bone extracellular matrix (ECM) proteins. Activation of the immune system and SA survival strategies coordinately affect the bone microenvironment (BME). This undergoes pathological alterations with formation of elusive niches (that favor chronic infection and antibiotic resistance) whose eradication requires invasive surgical interventions (bone debridement). A general consensus on OM pathogenesis, with major regard to the SA-bone cells crosstalk, is lacking, which poses severe limitations on diagnosis and

therapy of this highly debilitating disease.
We aim to fill this gap by investigating the mechanisms underlying the interaction of SA with the bone microenvironment. In particular, we will focus on the soluble pattern recognition receptor pentraxin 3 (PTX3), well-known for a wide range of functions in innate resistance to opportunistic pathogens, regulation of inflammation, wound healing, and recently emerging as a new player in bone homeostasis and pathology.

***Rational***

Strong evidence has been generated in our past investigations to support a role of PTX3 in the pathogenesis of OM. In particular:
1) we have established an animal model of OM based on injection of SA into the femur of mice. In particular, >95% of SA-treated mice (hereafter, WT SA and PTX3 KO SA) developed bone infection in the injected limb only (no bacteria were found in the contralateral limb), demonstrating that the infection was local (as is in the human pathology). Of note, WT SA mice had higher bacterial loads than PTX3 KO SA in the bone, suggesting that in this model the absence of PTX3 protected the experimental animals from SA infection. Accordingly, inflammation was more prominent in WT SA as compared to PTX3 KO SA. Also, PTX3 levels were higher in WT SA as compared to WT PBS both in the serum and bone, and most of the newly synthesized protein (during infection) localized in the infected bone (consistent with our clinical observations, see below). Finally, microcomputed tomography (microCT) analyses showed marked disruption of the bone matrix in the infected limbs; in this case too, structural alterations were more prominent in WT SA than in PTX3 KO SA;
2) we have developed a new 3D model of OM based on cocultures of SA and murine osteoblastic MC3T3-E1 cells on magnesium-doped hydroxyapatite/collagen I (MgHA/Col) scaffolds that closely recapitulate the bone extracellular matrix, and provide a novel and close-to-physiology tool to address the pathogenetic mechanisms of OM at the host–pathogen interface;
3) an observational study has been accomplished at the Ortho Center of HRH that aimed at assessing the accuracy of serum and synovial fluid (SF) PTX3 levels in the diagnosis of hip and knee periprosthetic joint infections (PJIs, a leading form of OM), in a cohort of patients undergoing prosthesis revision. Plasma levels of the protein were not affected by the infection, however SF levels were significantly increased in infected patients. In particular, SF PTX3 had an elevated diagnostic accuracy for hip and knee PJIs regardless of the infection's grade, whereas that of serum PTX3 was poor. In high grade PJIs, the diagnostic accuracy of synovial PTX3 was extremely elevated. Also, SF PTX3 had very high specificity both in high and low grade PJIs, indicating that this long pentraxin has a strong potential as a diagnostic biomarker in OM.

***Hypothesis***

Based on this rationale and our past experience with PTX3, we envisage that this long petraxin is strategically placed at the interface between infection immunity and bone biology, and holds promise as a target for innovative and original research into OM pathogenesis. Therefore, a funding hypothesis of this proposal is that PTX3 is involved in the molecular mechanisms underlying bone infection and colonization by SA, and OM development. More precisely, we hypothesize that this long pentraxin exerts multiple roles in OM as prototypical soluble molecule of the innate immune system (pattern recognition molecule, PRM) and component of the bone microenvironment (BME).

***General Objectives and specific aims***

To challenge this hypothesis, we propose an integrated research strategy that combines three levels of investigation: (i) animal experimentation (based on OM modelling in gene-modified mice), (ii) "disease-in-a-dish" approaches (using state-of-the-art 3D models), and (iii) clinical studies (to assess genetic and biochemical associations in patients with PJI). The general objective of the proposed investigations is to unravel molecular and cellular mechanisms of the OM pathogenesis, with a specific focus on the role of PTX3, as a prototypic soluble PRM and recently recognized player in bone pathophysiology.
To this ends, the following specific aims will be pursued:
1) to investigate the role of PTX3 in the etiopathogenesis of SA-dependent OM (SA-OM);
2) to identify cellular and molecular processes of the bone microenvironment that take place during SA infection and are possibly regulated by PTX3;
3) to define novel genetic profiles with diagnostic and prognostic potential in the clinical management of OM.

***Experimental Design***

A translational experimental strategy will be exploited that integrates in vivo modelling (in a murine model of SA-OM), in vitro experimentation

(on bone biomimetic 3D scaffolds), and clinical studies (in a cohort of PJI patients). Major tasks of the project proposal are outlined as follows:

1) Characterization of the molecular mechanism(s) of PTX3 in the pathogenesis of OM. Our previous investigations indicate that the absence of PTX3 is associated with reduced infection (and inflammation) in a murine model of OM. Following upon this evidence, we will extend the current protocol of animal experimentation to mice that are genetically or pharmacologically modified in other molecules (in addition to PTX3), including components of the complement system and/or of the extracellular matrix that are known to be recognized by PTX3 and are involved in bone physio-pathology. In this way, we expect to gather information on how PTX3 participates in the pathogenesis of OM, and, in general, on the molecular mechanisms of this disease. Parallel experiments will be performed in vitro using 3D bone scaffolds, which provide an easily controlled and tunable yet simplified model of the BME;

2) Evaluation of the pharmacological blockade of PTX3 and its combination with nano-delivered antibiotics in the prophylaxis/therapy of OM. Our past animal experiments suggest that the pharmacological blockade (for example, by means of antibodies) of the PTX3 protein could be a novel prophylactic/therapeutic strategy for treatment of OM. We will address this point, and assess efficacy and potency of PTX3 blocking antibodies (available in-house) in experimental murine models of SA-OM prophylaxis and therapy. Furthermore, we will develop new strategies for conjugation of SA-active antibiotics to nanoparticles and/or nanocarriers with the aim of facilitating their penetration into the bone matrix. We will, therefore, evaluate the combined effects of nano-delivered antibiotics and pharmacological blockade of PTX3 in the same murine models of OM. Similar experiments will be performed in vitro, whereby this setting is expected to support, guide and, possibly, extend the animal experimentation;

3) Assessing the diagnostic potential of PTX3 (and other selected genes) as a genetic biomarker in PJI. Our initial clinical observations strongly indicate that the levels of the PTX3 protein in the SF predict PJI in patients eligible for prosthesis revision with high specificity, thus pointing to this long pentraxin as a potential diagnostic biomarker in OM. To strenghten and extend this evidence, we have designed a retrospective genetic association study that has been recently approved by the Internal Review Board of HRH. In this study (which is expected to be completed in 2 years), patients are enrolled that took part in the previous protocol, genomic DNA is extracted from saliva specimens, and polymorphisms in the PTX3 gene (and other genes known to be involved in OM pathogenesis) are characterized. Along with available biochemical and clinical information (from the HRH's records), these (newly generated) genetic data will be integrated into prediction models to assess the diagnostic potential of PTX3 (and other genes) as a genetic biomarker of PJI, and, in general, identify a "genetic signature" that is associate with the risk of OM;

4) Validating the diagnostic potential of PTX3 (and other selected genes) as a genetic biomarker in PJI. We will perform a prospective multi-center genetic association study in an independent validation cohort of hip and knee prosthesis patients with a suspicion of PJI. Eligible patients (based on the criteria applied in the former study) will be enrolled over 4 years and donate blood and SF specimens (for DNA extraction, and dosing of the PTX3 protein). The genetic, biochemical and clinical information generated in the study will be processed and elaborated as described above to validate: i) the association between genetic variability in PTX3 (and other genes involved in the pathogenesis of PJIs) and susceptibility to the infection, and ii) the association between PTX3 protein levels in the SF and presence/absence of the infection and its severity.

***Expected Results***

Our study is expected to generate novel paradigms in the field of osteoimmunology, with major regard to the role of the interface between pathogen biology, innate immunity and bone microenvironment in the pathogenesis of bacterial bone infections. In particular, we propose the immune system and bone component PTX3 as a novel diagnostic and therapeutic target in SA-OM: to address this point, proof-of-concept experimental evidence will be provided both at preclinical and clinical level. Overall, we expect to: i) unravel the role(s) of PTX3 in SA-induced OM disease mechanisms, ii) identify characteristics of the bone microenvironment that are associated with susceptibility to OM, iii) provide proof-of-concept of possible therapeutic/preventive strategies, and iv) identify new genetic markers with diagnostic and prognostic value in the clinical handling of bone infections. Novel and original information will be delivered with valuable impact on health care systems, and benefit to the patients.

***Significance***

Our proposal is an interdisciplinary research program designed to create multi-faceted, cutting-edge new knowledge in the field of bone biology. To this end, it integrates technologies and methodologies of osteology, immunology, microbiology, protein biochemistry, and clinical diagnostics. In fact, our project will be implemented at 3 parallel yet integrated levels: (1) in vivo [tasks 1 and 2], in a mouse model of intrabone infection, whose use is justified by the mechanistic complexity of OM pathogenesis, (2) in vitro [tasks 1 and 2], by means of 3D cocultures of osteogenic cell lines and bacteria, and (3) per infirmos [tasks 3 and 4], through genotype-phenotype association studies in selected cohorts of PJI patients.

SA-OM is a hardly manageable bone infection, which easily becomes chronic, very painful and debilitating, often resulting into repeated surgeries or even amputation after failure of prolonged antibiotic therapies. Hence, the social and economic relevance of this pathology. In this regard, according to the 2019 annual report of the Italian Arthroplasty Registry, promoted by Istituto Superiore di Sanità (https://riap.iss.it/riap/it/), more than 70,500 arthroplasty surgeries have been performed in Italy in 2018; 5.6% of the total were revision surgeries, and in the 8.8 % of cases revision was required because of infection. Therefore, it is timely to develop new drugs for these complications, also to cope with the increasingly serious problem of multidrug resistant microorganisms. The functional characterization of the role of PTX3 in the specific pathological context of bone infections holds promise to have an important impact on public health and health expenditure, and lays the foundations for future development in the pharmaceutical setting.

Also, our study will broaden the knowledge on innate immunity in the framework of bacterial infections of the skeleton, and more in general on structural features of the bone tissue of relevance to this pathological context.

Finally, a junior PostDoc and a technician will be hired on the project, which will therefore provide a great formative opportunity for young researchers, and an added value for the development of their scientific and professional careers.