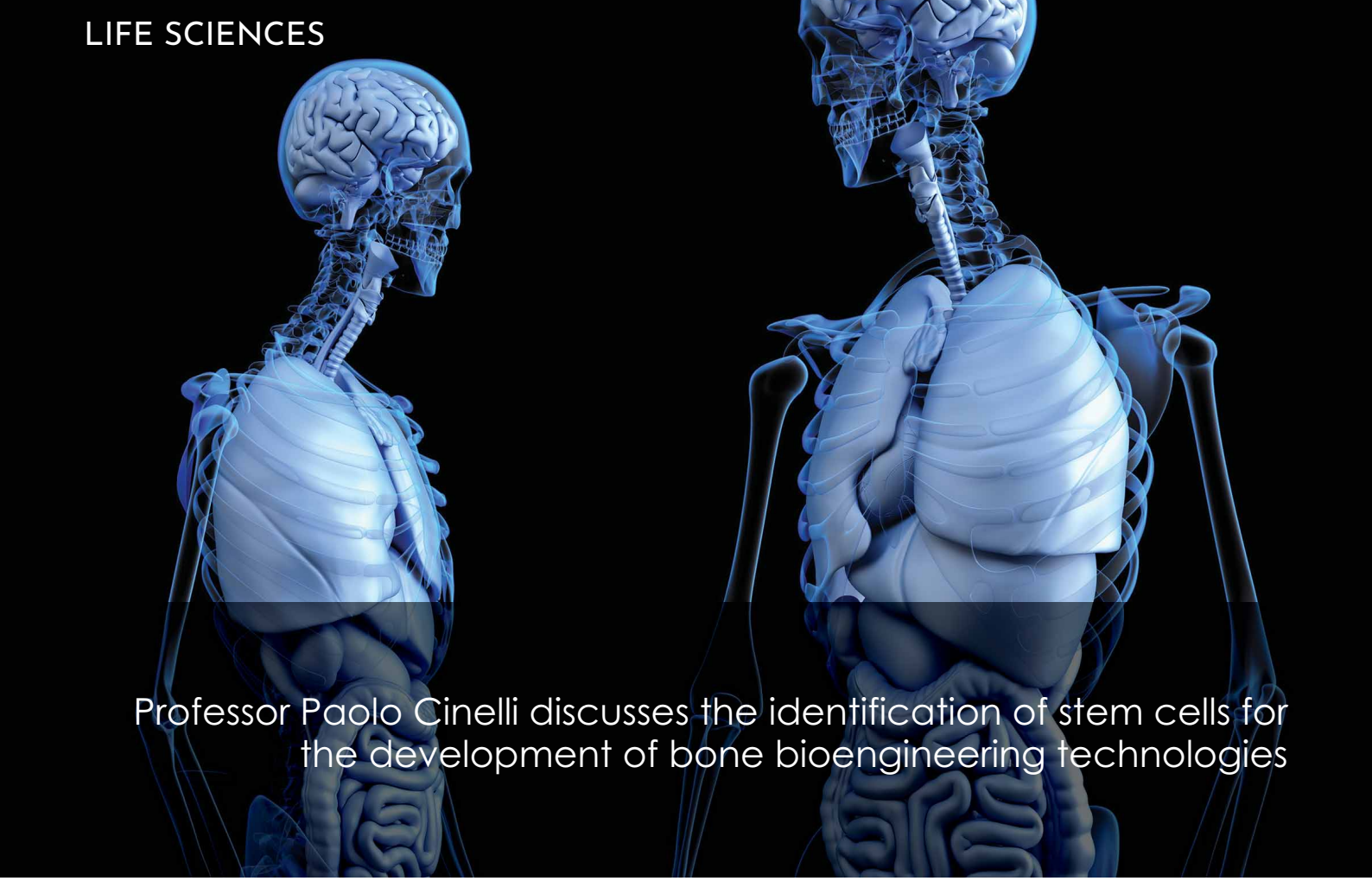

Professor Paolo Cinelli discusses the identification of stem cells for the development of bone bioengineering technologies

Bone: a dynamic organ

Bone is often considered as a rigid organ that provides support and physical protection to various vital organs of the body. In reality, bone is a very dynamic organ that is permanently in a dynamic balance, a process called 'remodelling', which allows a constant regeneration of bones. In the adult human body this tightly regulated remodelling process allows for the renewal of the entire skeleton every seven years.

Additionally, bone has also other important properties: it stores crucial nutrients, minerals, and lipids, and it produces blood cells that transport oxygen and play a vital role in protecting the body against infection.

Bone remodelling is only possible through a complex co-ordination of multiple bone marrow cell types: bone formation by osteoblasts and resorption by osteoclasts. An imbalance between bone formation and resorption can result in various diseases, such as osteopetrosis, osteopenia, and osteoporosis.

Bone fracture healing

One of the most fascinating properties of bone is that unlike in other tissues, the majority of bony injuries (fractures) heal without the formation of scar tissue, and bone is regenerated with its pre-existing properties largely restored and with the newly-formed bone being indistinguishable from the adjacent uninjured bone.

The process of fracture healing by intramembranous ossification and/or endochondral ossification also involves many events including the signalling, recruitment, and differentiation of bone marrow stromal cells during the early phase; the formation of a hard callus and extracellular matrix, angiogenesis and revascularisation during the mid-phase; and finally callus remodelling at the late phase of fracture healing.

Impaired healing

Despite the fine degree of orchestration during fracture healing, the process may be impaired. Currently, 10–15% of the fractures that occur annually result in poor or unresolved healing, so-called 'non-unions'. The mechanism of impaired osteoporotic fracture healing is multi-factorial and depends on the low sensitivity of osteoblasts to mechanical signals, reduced angiogenesis, and decreased amounts of mesenchymal stem cells. Particularly problematic and a major clinical orthopaedic challenge are the so-called 'critical size defects', which are bone fractures producing a gap that is not able to completely heal alone over a long period. In these cases, it is necessary to fill the non-union defect with dedicated materials or alternatively use strategies, which promote complete regeneration of the bone in these defects.

Nevertheless, current clinical treatments can be problematic and often yield poor healing due to the anatomy and physiology of bone tissue, as well as due to the limited knowledge about the bone healing process itself.

The gold standard

The gold standard is the transplantation of cancellous autogenous bone, a procedure that has some drawbacks for clinical applications, such as limited availability, morbidity, and donor site pain.

Skeletal defects may require volumes of bone that are often not available. Therefore, allografts are also used as substitutes for autologous bone grafts, but often are not sufficient to solve the many problems of bone deficiency. Alternatively, biocompatible materials can be used (e.g. calcium phosphate bioceramics) but, unfortunately, to date no single synthetic material offers all the benefits of the patient's own bone.

These materials can also be used in combination with osteoinductive factors like bone morphogenetic proteins (BMPs), transforming growth factor-α (TGF-α), and fibroblast growth factor (FGF). Due to the high number of limitations linked to the above-described procedures and the steadily increasing demand for bone grafting procedures, it is essential to develop better therapeutic approaches.

Bone tissue engineering

In this context, tissue engineering combining the use of stem cells with synthetic scaffolds and molecular signals (growth or differentiating factors) to form hybrid constructs represents a fascinating alternative.

Stem cells represent an appealing cell source for regenerative medicine applications aimed at improving bone fracture healing and treating bone-remodelling diseases like osteoporosis. Many reports have described the presence of mesenchymal stem cells in a variety of foetal, perinatal and adult tissues, including peripheral blood, foetal liver and lungs, skeletal muscles, amniotic fluid, synovium and the circulatory system where they contribute in maintenance of tissue homeostasis. These cells are able to differentiate *in vivo* as well as *in vitro,* under the right culture conditions, into osteoblasts, chondrocytes, and adipocytes.

In parallel to mesenchymal stem cells, a number of additional stem cell populations with pluripotent and multipotent properties exist which can potentially be used for bone engineering, such as embryonic stem cells, induced pluripotent stem cells, and adult neural crest stem cells.

Mesenchymal stem cells

In recent years, human fat tissue has been identified as a good source of mesenchymal stem cells, the so-called 'adipose-derived stem cells'. These are isolated from the stromal vascular fraction of adipose tissue, which contains pre-adipocytes, mesenchymal stem cells, endothelial progenitor cell, T- and B-cells, mast cells as well as adipose tissue macrophages. The amount of stem and progenitor cells found in the uncultured SVF from adipose tissue was estimated to be around 3% of the whole cells. This corresponds to 2,500-fold more stem cells than in the bone marrow. These cells represent therefore a good alternative to BMSCs for bone regeneration purpose.

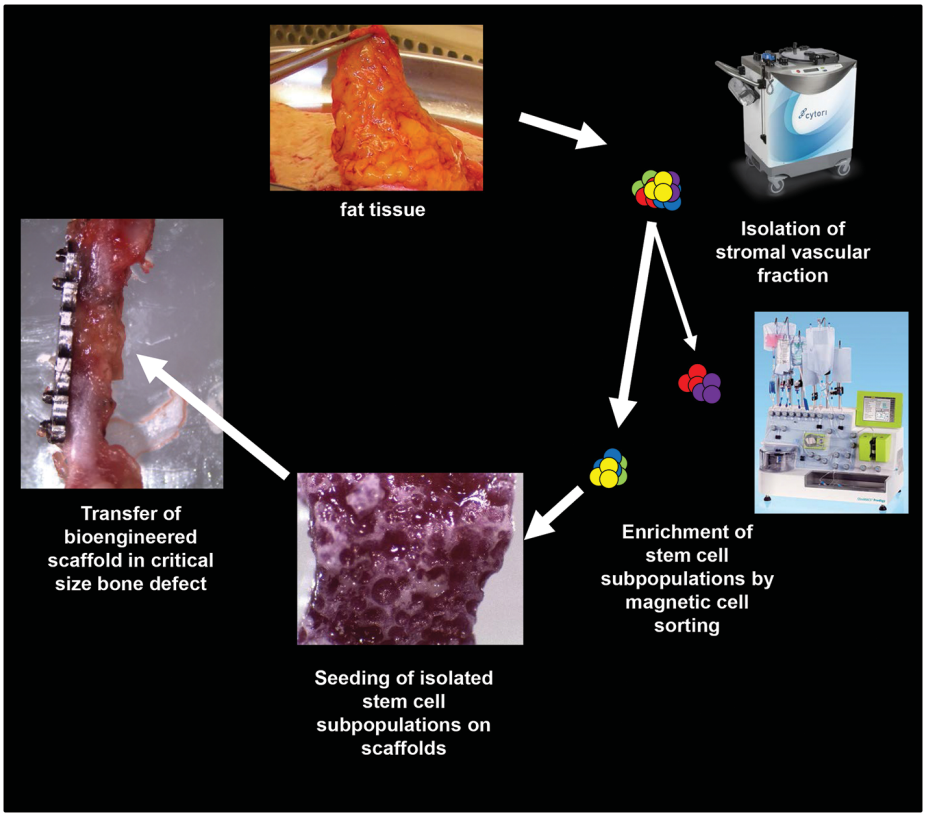

Fig. 2 In the adult human body the tightly regulated remodelling process, which allows a constant regeneration of bones, allows for the renewal of the entire skeleton every seven years

The main problem with mesenchymal stem cells of both bone marrow and fat tissue is that they consist of highly heterogeneous populations of stem and progenitor cells. Therefore, describing the entire culture as stem cells is inappropriate.

Moreover, several studies have reported differences in the expression of surface markers on mesenchymal stem cells and the currently-used markers are not a unique characteristic of mesenchymal stem cells; rather they reflect their heterogeneity. This variability represents a limiting factor for the efficient use of mesenchymal stem cells in the clinic. Thus, for the clinical use of mesenchymal stem cells for regeneration purposes, a better characterisation of the cells and a standardisation of the isolation and culture protocols are urgently required. To this end, it is mandatory to dissect at clonal level the composition of the different populations which are obtained with the current protocols.

Molecular mechanisms

Our laboratory has, for several years been interested in dissecting the molecular mechanisms involved in the maintenance of stem cell identity in different stem cell populations, like embryonic stem cells, induced pluripotent stem cells and mesenchymal stem cells. The goals of our projects are, in addition to the study of the molecular mechanisms underlying stem cell identity, to identify populations of multipotent stem cells which can be prospectively used for the development of bone bioengineering technologies.

Higher osteogenic potential

The isolation and analysis of mesenchymal stem cells subpopulations with higher osteogenic potential is also an important area.

The use of highly selective cell separation procedures in clinical cell-based treatments has the potential to improve the quality of repair and the subsequent clinical outcome. Nevertheless, the methodologies have to be adapted to a clinically compatible asset.

The cultivation of cells for therapeutic applications implicates the use of good clinical practice (GCP) facilities and long-time expansion of the cells *in vitro* before transplantation. It would therefore be interesting to develop a protocol in which subpopulations of stromal vascular fraction cells with a higher osteogenic differentiation potential could be enriched and employed without further expansion *in vitro* for clinical applications.

Magnetic activated cell sorting

An optimal solution would be the possibility of performing the enrichment directly in the operation room in parallel to the already necessary operative treatment of the patients (see Fig. 1). An interesting technology for this purpose is magnetic activated cell sorting (MACS).

In a recent study, we aimed at testing the feasibility of such an approach by using pericytes. Pericytes or vascular stem/precursor cells at

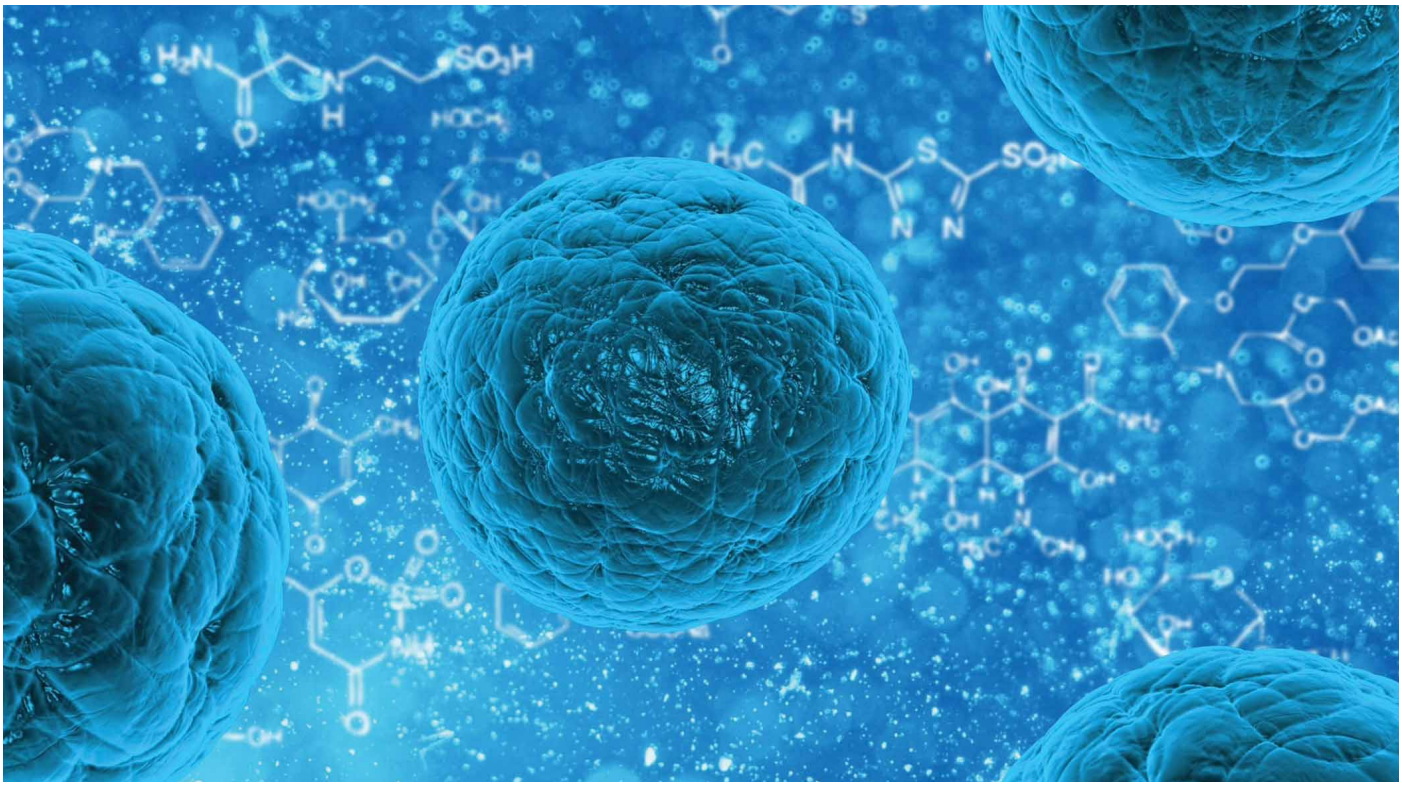

Fig. 3 Stem cells represent an appealing cell source for regenerative medicine applications aimed at improving bone fracture healing and treating bone-remodelling diseases like osteoporosis

different stages of differentiation are located in the wall surrounding the vasculature. Previous studies have identified markers typifying pericytes, in particular the surface markers NG2 and CD146 (also known as MCAM or S-endo1), which can be used to isolate pericytes upon depletion of the CD34 positive endothelial cell population and of the CD45 positive haematopoietic cells.

To this end, we have enriched CD146+NG2+CD45– pericytes from the stromal vascular fraction of fat tissue by magnetic sorting and tested their capacity, without any *in vitro* expansion, to take part in bone regeneration. We were able to show that enriched CD146+NG2+CD45– cells are able to differentiate to osteoblasts and induce calcium deposition (mineralisation) with a higher efficiency as unsorted adipose stem cells.

We further tested the regenerative capacity of the cells in a mouse model for femoral segmental critical-sized defect. In this model, a 3.5-mm-long segmental bone defect is induced in the mid-shaft of the mouse femur and bone fixation is performed with a titanium microlocking plate with four locking screws. The freshly isolated cells were seeded on a collagenous bone scaffold and the scaffold was inserted into the segmental bone gap.

Eight weeks after the operation the bones were isolated and following the removal of the plates the bone were analysed by microcomputer tomography and histologically. From this, we could confirm that direct transplantation of CD146+NG2+CD45– enriched cells from adipose tissue is sufficient to promote *in vivo* bone regeneration.

This study represents proof of principle for the use of enriched populations of cells with stem/progenitor identity for direct therapeutic applications and opens a new perspective for using stem cells in a clinical setting.

Stem cell maintenance

The identification of factors essential for the maintenance of stem cells. Pluripotent stem cells harbour a powerful potential for therapeutic application because they are able to generate every cell type of the body. Although it is possible to reprogramme somatic cells to a pluripotent state, the mechanisms underlying maintenance and determination of pluripotency remain unclear.

The same holds true for the mechanism driving the differentiation of these cells. This is not only the case for pluripotent stem cells but also in general for a large number of adult stem cells. Even though their identity is known, it often is impossible or difficult to maintain them outside of the body in a self-renewing pluripotent state.

Our laboratory has invested many efforts into the identification of new factors important for the maintenance of pluripotency and in analysing the mechanisms driving the reprogramming of somatic cells. We have, for example, elucidated the role of role of poly-ADP-ribose polymerase 1 (Parp1) during reprogramming and found that poly-ADP-ribosylation of the reprogramming factor Sox2 by Parp1 plays an important role during the first days upon transduction with the Yamanaka reprogramming factors.

We were also able identify Pramel7 (preferentially expressed antigen in melanoma like-7) as a novel factor crucial for LIF-mediated self-renewal in embryonic stem cells and recently show that Pramel7 targets UHRF1, a key factor for DNA methylation maintenance, for proteasomal degradation. This process leads to a gene signature and DNA hypomethylation comparable to the preimplantation epiblast, the developmental ground state and source of embryonic stem cells.

Our discovery revealed a previously unknown dynamic nature of DNA methylation through proteasome pathways and helps to improve embryonic stem cell culture to reproduce *in vitro* then *in vivo* ground-state pluripotency.

What is the true identity of mesenchymal stem cells?

A vast number of studies have identified a variety of cell surface markers expressed by mesenchymal stem cells in hopes of developing methods to isolate them more efficiently. However, these markers are not specific, either individually or in combination, and importantly their expression changes with time in culture. The real identity of mesenchymal stem cells is therefore largely unknown.

Nevertheless, this knowledge is of utmost importance to answer such basic questions as whether the trilineage potential of mesenchymal stem cells (osteogenic, chondrogenic and adipogenic differentiation) is an intrinsic property of a specific subtype of stem/progenitor cells or different types of stem/progenitors cells differentiate toward different fates. This information is not only essential for bone bioengineering but also for understanding the role of stem cells in diseases like osteoporosis.

Smart implants

Innovative and personalised orthopaedic implants are complex, requiring cross-disciplinary knowledge and understanding of processes such as 3D printing technology, computer modelling, and regenerative medicine, amongst other areas. Moreover, the current generation of implants have a limited lifespan – around 15 years – meaning that they need to be replaced up to twice in a patient's life through the means of revision surgery. As revision surgery is a complicated procedure, it can often lead to bone defects, whereby standard treatment to repair these bones involves using the patient's bone, or synthetic filler materials, in combination with medical implants. These complex procedures are long and can result in a high risk of infection, whether immediately after surgery or further down the line. Treatment for infection is also a challenging and extended process, which is often associated with hospitalisation and the incurrence of high healthcare costs.

In order to address problems with current implants, calls are being made for next-generation smart medical implants intended to accelerate healing whilst preventing infection. It is here that PRosPERoS (PRinting PERsonalized orthopaedic implantS) comes in, a project funded through Interreg VA Flanders, the Ministry of Economic Affairs, and Provinces Limburg (the Netherlands), as well as Flemish-Brabant (Belgium). PRosPERoS intends to develop personalised, smart, and biodegradable implants which are created through 3D printing on magnesium and zinc alloys. The process involves conducting a precision scan of the vertebrae with advanced imaging technology, as a result designing and printing implants in a way which is patient-centric.

Meanwhile, in an effort to reduce the high rates of osteoporosis in North Western Europe – leading to the greatest rates of fractures throughout all European regions – the BONE (Bio-fabrication of Orthopaedics in a New Era) project is looking to regenerative medicine to create smart implants. BONE aims to accelerate the uptake of cost-effective 3D smart implants, which are fabricated through electrospinning (ESP) technology. The technology supports regeneration of skeletal bone whilst replacing the demand for tissue donors, revision surgery, or lifelong medication schedules.

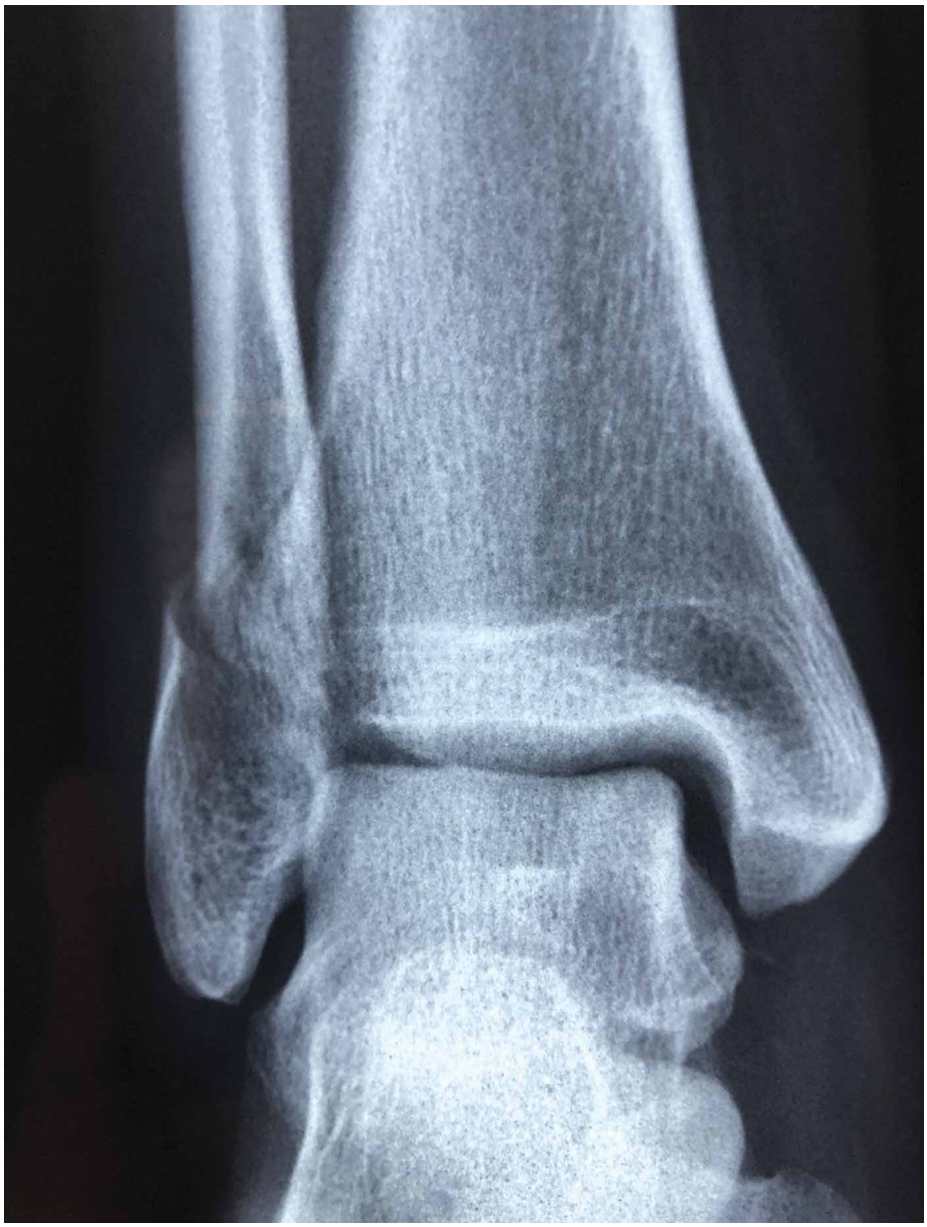

Fig. 4 Uncovering the real identity of mesenchymal stem cells is not only essential for bone bioengineering but also for understanding the role of stem cells in diseases like osteoporosis

We have recently developed a new tool based on the novel technology of cytometry by time-of-flight (CyTOF) to dissect the heterogeneity of human mesenchymal stem cells. With this tool, we are able to perform real-time analysis at high-dimensional level and at single cell resolution to detect the distribution and changes of markers within the heterogeneous population of mesenchymal stem cells.

In combination with state-of-the-art omics and imaging technologies we aim at dissecting the composition at single cell level of stromal vascular fraction cell populations and identify new specific markers for the prospective identification and isolation of selected stem cell populations for bone bioengineering.

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