THE NON-UNION TIMELINE: WHEN IS THE FATE OF A FRACTURE DECIDED?

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Even when fracture repair proceeds uneventfully, the considerable morbidity the patient experiences may be underestimated and it is often several years before full function has returned. However, if fracture repair does not progress smoothly and a non-union develops, the morbidity is substantially greater and often associated with severe financial hardship for the patient and a large burden for the healthcare system.

The current definition of non-union being a fracture that is un-united at nine months, should be considered a failure of modern fracture treatment.

The current long period before we diagnose non-union is a consequence of our inability to monitor healing in the first few months in the "forgotten phase" of fracture healing.

Various modalities have been tried for detecting non-union, which include imaging, mechanical and biomarker techniques. Unfortunately, none of these has yet demonstrated sufficient predictive power to be of general clinical use and it is has not been possible to determine when a failure of fracture healing has occurred.
This presentation discusses a possible framework for considering how best to monitor fracture healing in the forgotten phase ie the phase before healing is visible on X-ray.

Stem Cells For Bone Repair: A Clinical Perspective

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The “diamond conceptual framework” for bone regeneration denotes the minimum essential ingredients that must be present for a successful fracture healing response.

Osteoprogenitor cells are considered as the most important component of the “diamond concept”. Their importance is appreciated by their capacity to multiply, differentiate to osteoblasts, release molecular mediators and also participate in the processes of angiogenesis, and mineralization. Consequently, a lot of interest has been generated in the clinical setting as to how these small creatures can be used to develop regenerative therapies and to induce an environment of osteogenesis even in hostile environments.

Aspiration of bone marrow aspirate followed by centrifugation for cell concentration have been lately utilised substantially for hard tissue regeneration (bone and cartilage) including fracture non-union, osteonecrosis, osteochondral defects, etc. These cellular therapies can be either injected or loaded onto carriers (matrixes and scaffolds) particularly for the treatment of bone defects. However, the number of cells being implanted has been shown to be a limitation as the critical number of cells for a successful outcome has been calculated to be above 50,000. Consequently, focus has been given to such parameters that can affect the number of cells aspirated included the volume of aspiration (smaller the better), the diameter of the needle, the location of harvesting, and the speed of aspiration amongst others.

While expansion of cells has been used as the strategy to increase the number of cells implanted, such an approach is time consuming, costly and involves substantial regulatory scrutiny. Not surprisingly therefore, currently focus has been given to the isolation of fresh cells and their implantation at the point of care (surgery).

Different techniques of utilisation of MCSs will be presented for the treatment of different clinical conditions. Moreover, tips and tricks will be given on how to optimise outcomes.

Sharing a clinician's perspective on how clinical conditions can be treated successfully with researchers and scientists, will provide a solid foundation for a fruitful collaboration and exchange of ideas.

New innovations are on the way and more and more patients with difficult clinical musculoskeletal problems are expected to benefit out of these ever evolving advancements of using cellular therapies in bone repair.

Targeting Mechanosignalling to Modulate Bone Repair

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INTRODUCTION: Bone fractures represent a significant socioeconomic problem, with 5-10% of fractures requiring lengthy clinical interventions. Osteoporosis is a debilitating bone loss disease that leads to an increased risk of fracture and a compromised environment for bone repair resulting in further delayed/non-healing of fractures. This results in significant morbidity, mortality, and healthcare costs estimated at €36billion p.a. in the EU.

Mechanical loading is a potent stimulus promoting bone formation, and thus represents a potential stimulus to enhance the healing of delayed/non-healing fractures. Essential to continued bone formation is the differentiation of the stem/marrow stromal cell (MSC) population into bone forming osteoblasts. Therefore, this paper will discuss potential mechanisms by which mechanics can influence MSC differentiation and explore avenues by which these mechanisms can be targeted therapeutically to modulate bone repair.

METHODS: The transgenic mouse strain B6.129(Cg)-Lepr$^{tm2(cre)Rck}$/J was utilized to study and specifically target LepR-expressing MSCs in-vivo. 12-week-old female mice were subjected to compressive tibia loading with 11N peak load for 40 cycles, every other day for 2 weeks [1]. Bone cells were subjected to 2hr oscillatory fluid shear mechanical stimulation in-vitro using parallel plate flow chambers [2].

Extracellular vesicles were isolated and characterized as per ISEV guidelines [3]. Fibrous scaffolds were fabricated using Melt Electrowriting and were coated with a nano-needle hydroxyapatite coating [4].

RESULTS:

Firstly, a novel mechanotransduction mechanism in MSCs will be presented that is dependent on the cellular sub-compartment, the primary cilium. Deletion of the primary cilium in-vitro or in-vivo resulted in an inhibition of loading induced osteogenesis. Moreover, an orphan G-protein coupled receptor GPR161 was shown to localise to the cilium, and be required for mechanotransduction, introducing a potential novel mechanosensitive GPCR.

Secondly, this paper will also demonstrate an indirect mechanism whereby mechanically stimulated bone cells coordinate MSC recruitment and osteogenic differentiation in a paracrine manner. This mechanically activated secretome is shown to be multitargeted, also enhancing angiogenesis and inhibiting osteoclastogenesis. Interestingly, this mechanically activated paracrine signalling is mediated via the release of extracellular vesicles (EV), where EV regenerative cargo is enhanced in response to mechanical stimulation of the parent cell.

Finally, it will be shown that targeting these direct and indirect mechanisms of biophysical regulation of MSC behaviour represent novel mechanotherapeutic avenues to enhance MSC contributions to bone and new anabolic treatments for bone repair. These mechanotherapeutics can also be combined with bone mimetic fibrous scaffolds produced through Melt Electrowriting, resulting in novel mechano-biomimetic materials which have potential as an effective strategy to guide local bone regeneration.

DISCUSSION & CONCLUSIONS: Mechanics is a potent stimulus driving bone formation and repair and can act directly on cells such as inducing MSC osteogenesis via activation of mechanosignalling or indirectly via secreted factors such as extracellular vesicles released via mechanically primed parent cells. The delineation and targeting of this mechanosignalling represent new approaches to modulate bone repair.

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Bone Mechanoadaptation – local to whole-organ considerations

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Bone mechanoadaptation is thought to adhere to principles that predict straight shape, yet most bones have obvious curvature. This paradox may have endured as most in vivo loading studies examine only small segments of a bone rather than integrating these response at a 'whole-organ' level. Bone shape is governed by growth-related endochondral ossification and (re)modelling drift after birth that is deemed to be mechano-adaptively guided. Many questions regarding how loading influences whole-organ level bone shape are, however, unanswered. Does loading evoke changes that possess spatial hierarchy? How persistent are load-induced shape changes or are they, as mechanoadaptive principles predict, wholly reversible? Do mechanoadaptive changes in bone cell remodelling activity unerringly correlate with local tissue-level strains? Here, historical and new studies using a range of approaches will be re-explored to test this paradox and to address some of these questions. They will reflect briefly upon bones either derived from distinct embryonic origin or formed by intramembranous or endochondral ossification, the role of early embryo loading, and the possibility that skeletal tissues exhibit 'modular' rather than uniform relationships with mechanical load and also with osteotrophic factors (e.g. PTH). Focus will then shift briefly to the cellular basis of these responses, will consider how strain patterns differ with sex, and then how load history modifies the deficient load-related response linked to ageing. Finally, data will be presented from experiments using CT-based, 4-dimensional imaging and computational analysis to monitor acute and chronic whole bone shape adaptation and remodelling in vivo. Results confirm the expected reversibility of acute load-induced structural changes in some cortical regions, which adhere to strain magnitude-related regulation of (re)modelling and occur where load-induced anti-resorptive activity is initially focused. They also show, however, that loading drives significant and extensive changes in tibia shape and increases in mass that are lasting. These lasting changes were found to be independent of local strain magnitude and occurred where initial load responses were principally osteogenic. This indicates that loading stimulates nonlinear remodelling responses to strain that culminate in greater curvature, suggesting that whole organ level mechanoadaptation does not adhere with target strain-related principles, and that vast regions of the whole bone organ retain a lasting structural strain magnitude-independent load 'memory' where greater curvature is optimised for load predictability without sacrificing strength.

From basic research to patient benefit – The journey of the Biphasic Plate

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INTRODUCTION:

The importance of mechanical stimulation on bone healing is well-understood and has been studied for decades. Research results from Stephan Perren's "strain theory" or Lutz Claes' work on interfragmentary movement have become conventions among scientists and clinicians, but have, surprisingly, not yet made a profound impact on the daily clinical practice of fracture treatment, despite a persistent number of clinical healing complications and fixation failures caused by adverse mechanical healing environments and weak implants.

METHODS:

Motivated by a pressing clinical need as identified during a clinical immersion period, we aimed to render aforementioned research findings clinically assessable and developed the Biphasic Plate. This bone plate concept intrinsically controls interfragmentary motion by a simple design adaption, a transverse slot at the undersurface of the plate leading to a biphasic flexion behaviour with a flexible and a stiff phase. The concept intends to provide a desirable amount of interfragmentary strain for optimized fracture healing independent of the patient's weight bearing capacity. At the same time, plate strength is significantly increased to allow immediate full weight bearing and avoid implant failures. The concept pays highest attention to simplicity and usability in current clinical practise. Complexity under real-world conditions is a reason why many innovations fail despite undisputed theoretical potential.

RESULTS:

After computational- and bench feasibility testing a first Biphasic Plate prototype proved the potential of the concept in animal testing exhibiting a significantly larger and stronger fracture callus compared to a control group under various conditions in vivo [1]. A human version was then developed indicated for distal femur (DF) fracture fixation under consideration of the regulatory requirements for a Class IIb implantable medical device [2]. CE mark was granted in 2021 for introduction of the plate to the European market. The first patient was operated in March 2022. Furthermore, in December 2022 the Biphasic Plate obtained AO approval by the AO Technical Commission. Currently a focused clinical registry is ongoing to investigate the clinical value of the concept. First results are promising, including a revision case with an established non-union, which healed uneventfully after reoperation with a Biphasic Plate DF.

CONCLUSION:

Direct or indirect translation of research findings into patient benefits is the overarching aim of research. The Biphasic Plate is an example of such translation process. The actual extent of the clinical potential will crystallize in the near future.

Biphasic Plate DF

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Subchondral bone migrates into trabecular bone in response to direct bone-on-bone contact wear in patients with severe knee osteoarthritis.

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INTRODUCTION: Although it is well established that osteoarthritis (OA) is accompanied by changes in subchondral bone microstructure, such as cortical thickening, little is known about microstructural adaptations at and around full-thickness cartilage defects in severe knee OA. This high-resolution microCT study therefore aimed to describe and quantify subchondral bone changes due to wear under direct bone-on-bone contact in patients with severe OA.

METHODS: Ten tibial plateaus showing full-thickness cartilage defects were retrieved from total knee arthroplasty patients. All patients provided signed informed consent. Six 5x5 mm specimens were cut with a diamond saw from the defect, defect-edge, and cartilage region and two from the contralateral side. These were microCT-scanned at a resolution of 3.3 µm. Bone volume fraction (BV/TV) and Tissue Mineral Density (TMD) were analysed layer-by-layer to compute the specimens' density profiles and identify cortical and trabecular bone regions. Bone formation around cortical porosity was derived from the gradient of mineral density around the pores.

RESULTS: The subchondral bone plate below full-thickness cartilage defects appears to migrate into trabecular bone through direct thickening and through pockets of bone formation within the trabecular structures. In cases with deep wear grooves, trabecular pores occasionally connect with the joint space before they are completely filled with new bone. The spatial expansion of these effects shows a sharp boundary at the defect-edge, almost immediately disappearing under regions covered with even a minimal layer of cartilage (Fig. 1). Quantitatively, cortical bone was 4-fold thicker below defects than below cartilage. BV/TV and TMD profiles significantly differed between defect, edge, and cartilage specimens, up to 5mm deep. Below defects, cortical porosity and trabecular BV/TV were 85% and 14% higher and TMD 6% lower compared to cartilage specimens. The TMD gradient around pores was greatest in the edge specimens.

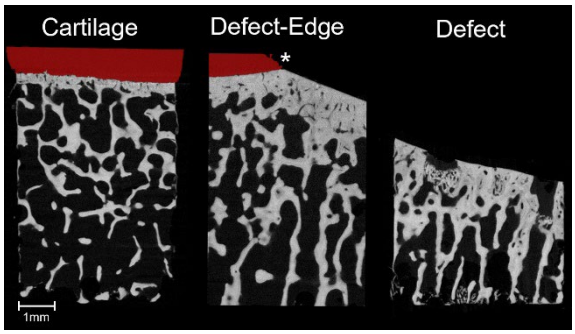

*Fig. 1: MicroCT images of bone specimens, from left to right: cartilage, edge defect and defect specimen. The specimens are oriented with the joint space at the top and the level of the surgical cut at the bottom. Cartilage is enhanced in red. The * marks the start of the defect in the defect-edge specimen.*

DISCUSSION & CONCLUSIONS: This is the first study to bring new insights into bone microstructural adaptations below full-thickness cartilage defects in severe knee OA. We observed subchondral plate migration below cartilage defects via trabecular osteogenesis. Our data shows an increased bone formation in defect and edge regions, reflecting the wear of native cortical bone and trabecular corticalisation, causing the cortical layer to migrate down into the trabecular bone.

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Mechanical strain triggers naïve MSCs in vitro differentiation towards hypertrophic chondrocytes.

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INTRODUCTION: The ultimate outcome of bone healing greatly relies on the initial mechanical conditions of the fracture [1]. In vivo, complete stability and an interfragmentary strain (IFS) below 2% are necessary for direct bone healing [2]. In all other cases, mostly endochondral ossification takes place, in which the frequency, and magnitude of IFS play crucial roles. Yet, the specific impact of these parameters on cellular-level regulation remains uncertain and understanding the regulation of naïve human mesenchymal stem cell (MSC) differentiation upon mechanical stimuli is vital for the development of efficient strategies for bone healing.

METHODS: Ficoll isolated bone marrow derived human MSCs (KEK ZH NR 2010 0444) from 5 donors were used. Cells of passage 2 to 3 were embedded in 8% GelMa hydrogels at a density of 20 x 10^6 cells / cast in custom made moulds 5 mm Ø x 4 mm) in the presence of 0.15% LAP photo initiator (Gelomics) and exposed to visible light for 8 min [3]. A custom made multi well bioreactor was used to apply a defined uniaxial load (StrainBot, RISystem, CH). The samples were exposed to 0, 10 or 30% of strain for 5 seconds, followed by 2 hours break, 24 hours per day for 14 days. Deformation protocols were performed in the presence of DMEM high glucose, containing 10 µm ITS, 50 µg/mL ascorbic acid, 1 NEAA, 100 mM Dex with or without exogenous 2 ng/mL TGFβ1. Cell differentiation was assessed by gene expression analysis, GAG analysis, immunostaining for type 1, 2 and 10 collagens, and Safranin-O/ fast green staining. Statistical analyses were performed using either ANOVA with Tukey's post-hoc correction or the Kruskal-Wallis test with Dunn's correction, depending on the data distribution.

RESULTS: Data showed a strong upregulation of hypertrophic related genes COMP, MMP13 and type 10 collagen upon stimulation when compared to chondrogenic SOX9, ACAN, type 2 collagen or to osteoblastic related genes type 1 collagen and Runx2. When compared to chondrogenic control medium, cells in CP with or without stimulation showed low proteoglycan synthesis as shown by Safranin-O/ fast green staining. In addition, the cells were significantly larger in 10% and 30% strain compared to control medium with 0% strain. Type 1 and 10 collagen immunostaining showed stronger collagen 10 expression in the samples subjected to strain compared to control. No clear classical chondrogenic differentiation (type 2 collagen, GAG production) was observed in the presence mechanical deformation alone. The addition of 2 ng/mL TGFβ1 induced an increased type 2 collagen gene expression.

DISCUSSION & CONCLUSIONS:
Our findings consistently suggested that naïve MSCs undergo hypertrophic chondrocyte differentiation when subjected to uniaxial deformation for a duration of 14 days without TGFβ1. We observed a strong presence of the hypertrophic markers type 10 collagen and MMP13, as well as other indicators of hypertrophic chondrocytes, such as reduced deposition of GAG and an increase in cell volume. Traditionally, in vitro models for MSC hypertrophic-chondrocyte differentiation involves the use of TFGβ and BMP2 along with 3D micro-mass cultures to first promote differentiation of chondrocytes expressing type 2 collagen and producing GAG under Sox9 regulation. Additional BMP6 and/or Dex treatment further triggers maturation towards hypertrophic chondrocytes expressing type 10 collagen and MMP13.
In our in vitro system, we found that naïve MSCs embedded in 3D GelMa hydrogels were able to develop a hypertrophic chondrocyte phenotype solely under the influence of mechanical uniaxial loading, without the addition of any growth factors. This phenomenon was also observed by other researchers studying MSC pellets, where an early onset of type 10 collagen expression was described [4]. Uniaxial deformation alone seems to be sufficient to promote in vitro hypertrophic-chondrocyte differentiation of naïve MSCs.

ACKNOWLEDGEMENTS: Funded by the AO Foundation, StrainBot sponsored by RISystemAG & PERRENS 101

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Biomimetic Hematoma to Enhance Bone Healing: Current Status and Future Clinical Implications

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The incidence of fractures amount to approximately 180 million per year worldwide. While bones have an innate ability to heal on their own, there is an entire subset of fractures (about 15%) that cannot heal and often require multiple surgeries. Furthermore, large bone defects, as a consequence of infections, tumors, trauma, or revision surgery, affect not only long bones, but also the skull and spine. Many techniques have been employed for the treatment of skeletal defects, including filling the void with a mix of natural and synthetic substitutes. These orthobiologics have only met with limited success because the procedures are painful, have a high risk of treatment failure, and have high complication rates, resulting in an enormous economic burden with a high risk of amputation. The most promising option has been bone morphogenetic protein -2 (rhBMP-2), which is currently the most potent inducer of bone formation. It is sold as a product called INFUSE™ (Medtronic Inc.), but due to its many associated deleterious side effects it is only approved for spinal fusions and open tibial fractures. The main problem with rhBMP-2 is that it is delivered at an extremely high dose via a collagen sponge/scaffold, most of which rapidly leaches away as soon as the collagen sponge becomes compressed after insertion. Moreover, uncontrolled delivery can also lead to other local problems and complications. Clearly, a much more controlled delivery vehicle is required for the precise delivery of growth factors such as rhBMP-2.

Hematoma (blood/fibrin clot) normally formed at the fracture site significantly influences the way fractures heal. The structural properties of a formed fibrin clot, such as porosity and thickness of fibrin fibers, influences bone repair, and the removal of this hematoma delays fracture healing. Therefore, the rationale was to create a natural scaffold, a Biomimetic Hematoma (BH), to mimic innate fracture hematoma that acts as a carrier to effectively deliver rhBMP-2. A structurally well-organized BH also serves as a temporary reservoir for the continuous release of growth factors, and it provides a suitable environment that encourages cell infiltration, proliferation, and differentiation from the surrounding tissues.

This was achieved by using a precise mix of calcium and thrombin combined with whole blood, creating an ex vivo BH that replicates the intrinsic structural and biological properties of innate healing fracture hematoma to deliver growth factors such as rhBMP-2 for a more efficient treatment of complex fractures. In essence, this method allows surgeons to create a hematoma using the patient's own blood to insert into the bone defect that then gradually releases these factors, promoting faster and more robust healing. Preclinical small and large animal studies, as well as a small preliminary clinical study, have already demonstrated that BH is currently the only known carrier able to effectively deliver much lower doses of rhBMP-2 with high efficiency, consistently and robustly initiating the bone repair cascade to successfully reconstruct complex bone fractures without side effects. Healing is initiated in these studies with an 80-90% reduced dose of rhBMP-2 compared to the lowest effective dose previously used with any other biomaterial scaffold. This novel patient specific treatment should dramatically improve the management of complex bone injuries, thereby decreasing the impact of persistent non-healing bones in severely injured persons. So far, no deleterious side effects were identified when using BH to deliver rhBMP-2, which were common when using INFUSE™. The preparation of BH can be done on demand intra-operatively, very quickly, reproducibly, and economically, even in austere environments using either patient or donor blood.

Biomimetic Hematoma is a natural carrier that is able to deliver a low dose of BMP-2, or other biologics, to enhance robust and efficient bone healing, without side effects, for complex bone injuries. This natural product holds genuine promise to become a widely used treatment strategy, as it is an efficacious and safe solution for a wide variety of orthopedic, maxillofacial, dental, and veterinary applications.

External Biomechanical Stimulation as An Approach to Stimulate Fracture Healing

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Fracture healing is a complex and sequential process, while osteoporotic fracture healing is usually impaired and delayed. In recent years, this is known most of fragility fracture patients (~70-90%) have sarcopenia and reported that sarcopenia is associated with poor fracture healing outcomes. Therefore, any intervention that can enhance the healing process and improve muscle rehabilitation simultaneously would be most advantageous for osteoporotic fracture healing.

Mechanical stimulation is known to be a good intervention for musculoskeletal system. We have developed a low-magnitude high-frequency vibration (LMHFV; 35Hz, 0.3g, g=gravitational acceleration) treatment for osteoporotic fracture healing, where its effects on the entire healing process from mechanotransduction, inflammation, callus formation, and remodelling were validated in vivo comprehensively with shortening of healing time by around 30%. In the meantime, the efficacy of LMHFV treatment on skeletal muscle and balancing ability were confirmed in a randomized controlled trial involving 710 community elderly with reduced fall rate by 44%, whereas its effect on sarcopenia was also depicted in vivo. In 2023, LMHFV is recommended by Centres for Disease Control and Prevention (CDC) as an effective intervention for fall prevention in older adults (https://www.cdc.gov/falls/programs/compendium.html).

With the accumulative evidence, our developed LMHFV was granted with a PCT patent and successfully licensed to commercial use. Therefore, LMHFV is of good potential to be translated for clinical application to enhance fracture healing. Our translation work is on-going, which a randomized controlled trial is undergone to confirm its efficacy. Our ultimate goal is to translate LMHFV in trauma applications so that the fragility fracture patients will be benefitted.

Clinical trials on expanded MSCs for bone regeneration

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INTRODUCTION:

Bone healing may be altered both in trauma and orthopaedic injuries and diseases. Bone healing impairment may produce significant clinical, social and economic impact due to disability and functional limitations, repeated hospitalization and significant resource consumption. The surgical treatment frequently needs to incorporate bone augmentation, the standard being the bone autograft, with potential complications at the donor site, and with limitations due to insufficient stock.

BONE HEALING IN NON-UNIONS:

Delayed union and particularly non-union after fracture (when bone healing is not completed 9 months after the fracture, with absence of progressive signs of healing on serial radiographs over the course of three consecutive months) suppose challenging scenarios for bone healing. Biological augmentation in the treatment of non-union, particularly if recalcitrant, requires alternative approaches to bone autograft to improve the treatment effectiveness. The clinical application of mesenchymal stromal cells (MSCs) have been proposed but clinical results are scarce. Through different EU-funded projects, our consortium has implemented and evaluated the safety and efficacy of expanded autologous bone marrow (BM) derived MSCs in the long bone non-unions. The combination of biomaterials and autologous expanded BM-MSCs under good manufacturing practices (GMP) has been successfully implanted in early trials (Gomez-Barrena et al 2019) at a dose of 20 million MSC/cc. Furthermore, a randomized clinical trial between MSCs (at high and low doses of 200 million and 100 million cells) versus iliac crest autograft as a control has been completed, with early confirmation of efficacy. The evaluation of bone healing, the clinical results and the imaging and 3D bone regeneration will also be discussed in this presentation.

OSTEONECROSIS REGENERATION:

Osteonecrosis (ON) of the femoral head is a potentially severe hip disease due to insufficient bone regeneration after the onset. This lack of bone regeneration may lead to femoral head collapse and secondary osteoarthritis, with serious pain and disability. Intents to foster bone regeneration in osteonecrosis focused on the administration of MSCs through the forage, if performed at earlier stages of the disease. In view of the limitations and variability of bone marrow concentrate techniques, our group has completed a multicentric phase II open clinical trial proving safety and efficacy to heal early femoral head ON in about 80% patients with 5 years follow-up. Furthermore, the location of the forage was analysed to understand the healing potential of the technique. Three-dimensional imaging analysis of the regenerated bone offered new insights about the strengths and limitations of this technique.

CONCLUSIONS:

MSCs in the treatment of long bone non-unions and early osteonecrosis of the femoral head still require substantial research, but bone regeneration has been proven feasible, safe and efficacious, orienting future directions.

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A Novel Bandage For Bone Repair

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Introduction: Bone defects pose significant challenges for patients and healthcare systems, often requiring extensive donor bone tissue. Cell-based therapies using human skeletal stem cells (hSSCs) offer a promising approach, but their low survival rate remains a concern. Understanding the mechanisms governing hSSC function is crucial for addressing this issue. Hydrophobic Wnt proteins, often secreted locally and activate Wnt/β-catenin pathway, play a vital role in hSSC self-renewal. Conventional activation of the Wnt/β-catenin pathway in vitro and in vivo is limited to soluble Wnts in detergent or lipid micelles, or membrane-permeable small molecules. However, global addition of Wnts or small molecules provides non-directional signals, leading to cellular responses that can significantly differ from those observed in tissues [1].

Methods: We developed a novel approach by covalently binding Wnt3a onto microbeads [1] and an aldehyde-modified glass platform [2,3], enabling localized presentation of Wnt signals to cells. This Wnt3a-platform activated the Wnt/β-catenin pathway in hSSCs, promoting long-term self-renewal. We extended this concept to three-dimensional (3D) osteogenic structures, culturing hSSCs on the Wnt3a-platform within a collagen type 1 gel (COL1-gel) environment.

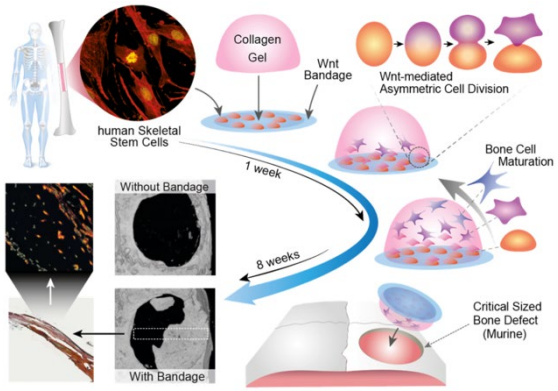
Results: Within 7 days, we observed the formation of an organized three-dimensional Wnt-induced human osteogenic tissue model (WIOTM). The WIOTM consisted of Wnt3a-proximal hSSCs and a multilayer of differentiating cells, exhibiting markers of osteogenic differentiation as they migrated away from the Wnt3a source. Mineralized nodules were observed in the upper regions of the WIOTM COL1-gel, effectively recapitulating a physiological human bone niche in vitro. Further characterization revealed asymmetric stem cell division as a driver for WIOTM formation and confirmed the presence of specific cell populations using recently identified cell fate markers.

Next, we designed a bandage, the WIOTM-bandage (Figure 1), to deliver the WIOTM to critical-sized defects in murine calvaria. Evaluation of in vivo

WIOTM survival and its contribution to bone formation demonstrated the maintenance of hSSCs and mature human bone cells even after 8 weeks. Importantly, the WIOTM-bandage exhibited enhanced endogenous bone repair. The newly formed bone displayed structural similarities to mature cortical bone, containing both human and murine cells and a functional vascular network [4].

Conclusion: Our study sheds light on the control of human osteogenesis and presents a promising strategy for delivering viable human osteogenic constructs capable of surviving in vivo and contributing to bone repair. The localized presentation of Wnt3a holds significant potential in improving bone regeneration therapies.

Figure 1: WIOTM-bandage for bone repair



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The interplay of angiogenesis and osteogenesis for bone regeneration

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Bone regeneration is an area of acute medical need, but its clinical success is hampered by the need to ensure rapid vascularization of osteogenic grafts for their survival and proper tissue formation. Vascular Endothelial Growth Factor (VEGF) is the master regulator of vascular growth and during bone development angiogenesis and osteogenesis are physiologically coupled through so-called angiocrine factors produced by blood vessels. However, how to exploit this process for therapeutic bone regeneration remains a challenge (1).

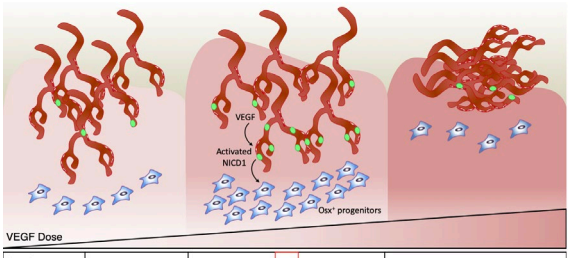
Here we will describe recent work aiming at understanding the cross-talk between vascular growth and osteogenesis under conditions relevant for therapeutic bone regeneration. To this end we take advantage of a unique platform to generate controlled signalling microenvironments, by the covalent decoration of fibrin matrices with tunable doses and combinations of engineered growth factors. The combination of human bone-marrow derived osteoprogenitors and hydroxyapatite in these engineered fibrin matrices provides a controlled model to study the role of specific molecular signals in the regulation of vascular invasion and bone formation *in vivo*. In particular, we found that:

1) Controlling the distribution of VEGF protein in the microenvironment is key to recapitulate its physiologic function to couple angiogenesis and osteogenesis (2);

2) Such coupling is exquisitely dependent on VEGF dose and on a delicate equilibrium between opposing effects. A narrow range of VEGF doses specifically activates Notch1 signaling in invading blood vessels, inducing a pro-osteogenic functional state called Type H endothelium, that promotes differentiation of surrounding mesenchymal progenitors. However, lower doses are ineffective and higher ones paradoxically inhibit both vascular invasion and bone formation (Figure 1) (3);

3) Semaphorin3a (Sema3a) acts as a novel pro-osteogenic angiocrine factor downstream of VEGF and it mediates VEGF dose-dependent effects on both vascular invasion and osteogenic commitment of progenitors.

In conclusion, vascularization of osteogenic grafts is not simply necessary in order to enable progenitor survival. Rather, blood vessels can actively stimulate bone regeneration in engineered grafts through specific molecular signals that can be harnessed for therapeutic purposes.



Invasion	+		+		-
Notch1 Activation	-		+		-
Osx+ Progenitors	-		+		-

Figure 1. VEGF dose regulates vascular invasion, Notch1 activation and osteogenic commitment of human progenitors in engineered grafts (from Grosso, Lunger, Burger et al. *npj Regen. Med.* 2023).

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Effects of low dose BMP-2 on cytokine levels during fracture healing in a femur segmental defect in rats

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INTRODUCTION: Effective bone healing relies on a robust but timely-restricted pro-inflammatory phase[1]. Clinically applied bone morphogenetic growth factors like BMP-2 show good healing capacity but have been associated with excessive and prolonged pro-inflammatory cytokine release and heterotopic ossification in muscle at high doses[2]. This study aims to investigate the effect of low dose BMP-2 (1 µg) on callus formation and cytokine levels in the fracture repair tissue (haematoma, callus) and adjacent muscle in a femur segmental defect model in rats.

METHODS: 2 mm segmental femoral defects were created in skeletally mature (19-24 weeks old) female F344 rats (n=3 per group), internally fixed with a 1.25 mm-thick polyetheretherketone plate (animal license: GR/19/2022 (nat. number: 35156)) using established protocols for analgesia and anesthesia. Animals received either no treatment (empty defect), a Lyostypt collagen sponge, or Lyostypt collagen sponge + 1 µg BMP-2 (InductOs, Medtronic). Animals were sacrificed at either 3, 7 or 14 days, the fracture repair tissue, as well as the adjacent muscle, were collected, snap frozen in liquid nitrogen, and stored at -80°C. Afterwards, the tissues were collected in T-PER tissue protein extraction reagent (Thermo Fisher) and the levels of interleukin 1β (IL-1β) were determined by ELISA (R&D Systems DuoSet). Radiographs were taken at surgery, and at regular intervals throughout the study to determine healing efficacy.

RESULTS: IL-1β levels in the fracture repair tissue on post-operative day 3 were significantly higher in the collagen (48%; p=0.001) and collagen with BMP-2 group (50%; p=0.001) compared to empty controls, while a peak was found for all groups on day 7, followed by a sharp decline until day 14. Lower IL-1β levels were detected in the muscle than in the fracture repair tissue. Radiographs demonstrate that collagen scaffolds with 1 µg BMP-2 induced pronounced new bone formation and cortical bridging 2 weeks post-operatively.

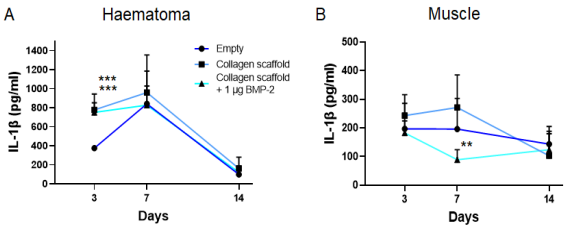

*Fig. 1: IL-1β-concentration in (A) haematoma and (B) adjacent muscle on days 3, 7 and 14 of fracture healing. Data represents the mean ± standard deviation (n=3);***indicates p<0.001 (compared to empty group), ** indicates p<0.01 (compared to collagen group), one-way ANOVA.*

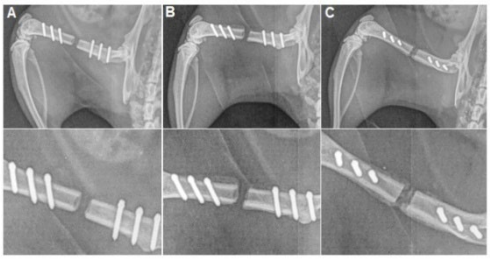

Fig. 2: Representative radiographs of femoral defects 2 weeks post-surgery. (A) Empty, (B) Collagen sponge, (C) Collagen sponge + 1 µg BMP-2.

DISCUSSION & CONCLUSIONS: Low dose BMP-2 had a strong local effect on new bone formation during fracture healing in a femur segmental defect models in rats while maintaining IL-1β levels in the adjacent muscle comparable to controls. Future work may explore the potential of immunomodulation strategies to counteract excessive and prolonged cytokines levels associated with clinically applied higher doses of BMP-2.

ACKNOWLEDGEMENTS: This project was supported by AO foundation. The author's acknowledge Andrea Further for performing *in vivo* micro-computed tomography scans.

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Filling the Gaps: Dynamic Alginate-Hydroxyapatite Composite for Bone Tissue Regeneration

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INTRODUCTION: Alternatives to the gold standard autografts are critical to heal critical sized bone defects. Even though synthetic bone graft substitutes are commercially available, they still lack important biological competence. Producing a vascularized bone graft substitute that promotes cell migration and differentiation is crucial to restore native bone structures on large defects. We propose a dynamic bone graft composite made of hydroxyapatite embedding alginate beads carrying out a double function: dynamically creating additional porosity for cell and fluid invasion and encapsulating human mesenchymal stromal cells (hMSCs). After degradation of the beads, the released hMSCs will subsequently differentiate into osteoblasts in the defect.

METHODS: Oxidized alginate beads encapsulating hMSCs were prepared by electrospraying in a calcium chloride bath. Beads were obtained from alginate with degrees of oxidation of 2.5%, 5% and 10%. Bead sizes were controlled by pressure, voltage, and the needle gauge used during electrospraying. A mixture of the obtained beads was prepared with a calcium phosphate (CaP) phase (tetracalcium phosphate (TTCP) and dicalcium phosphate, anhydrous (DCPA)) as well as an alginate solution buffered to maintain the physiological pH to form a stable crosslinked alginate-hydroxyapatite matrix with a liquid-to-powder (L/P) ratio of 3/5. The hMSC-beads-CaP composites were then moulded into spheres and incubated in phosphate buffer until further use.

RESULTS & DISCUSSION: To study the degradation rates of the beads, they were fluorescein-tagged and the fluorescence intensity in the supernatant was followed over 12 days. Oxidized alginate beads degraded within days contrary to non-oxidised ones. Gradual degradation of the beads and release of the encapsulated cells was detected for the oxidized alginate beads while the non-oxidised alginate beads remained intact. The corresponding cell release and the cell morphology were observed by microscopy with Phalloidin/DAPI staining (Fig. 1). The cells released from the beads on tissue culture plate (TCPS) were elongated as opposed to the cells in the beads, smaller and rounder. Cells seemed to continue proliferating either in the beads or on TCPS over the course of 9 days. This could be explained by the interaction of the neighbouring cells in the beads, owing their slight elongation rather than being round like on day 1.

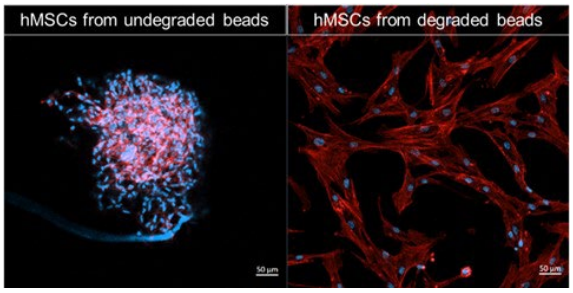

Fig. 1: hMSCs morphology before (left) and after (right) bead degradation on day 9. Scale: 50 µm.

Bone grafts formulations with calcium phosphates were optimized to prepare a cohesive material in static aqueous conditions stable for at least 21 days. Upon immersion of the composite material in solution, hydroxyapatite is produced, while sodium alginate forms a water insoluble gel in the presence of calcium ions mainly provided by TTCP, resulting in the stability of the graft. The studies also showed good moulding capacity of the grafts with and without the beads, showing potential to fill large irregular gaps.

CONCLUSION: These multiphasic composites provided dynamic properties for cell release and dynamic porosity, preserving structural integrity. Further investigations are necessary to determine their efficacy as bone graft substitutes.

ACKNOWLEDGEMENTS: The AO Foundation and the European Union's Horizon 2020 research and innovation program under grant agreement No 857287 for financial support.

A TGF-β cross-talk mechanism, in both breast and prostate cancer, which disrupts osteocyte primary cilia-mediated regulation of bone metastases

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INTRODUCTION: Bone is one of the most common sites for tumor metastasis, with 75% of breast cancer patients developing skeletal lesions[1,2]. This study examines the role of the osteocyte primary cilium, a key mechanosensor in bone cells, in regulating breast and prostate cancer cell phenotype, and cross-talk between cancer cells and osteocytes.

METHODS: Conditioned media from MLO-Y4 osteocyte-like cells (1:1 ratio) inhibited proliferation of breast (MDA-MB-231 & MCF-7) and prostate (PC-3 & LNCaP) cancer cells over 48hrs, while co-culture had no significant effect (Fig1). A series of cytokine arrays and ELISAs identified TNF-α secretion as a potential anti-proliferation mechanism inherent in osteocytes. We also found that TGF-β, secreted by cancer cells, reversed these effects by knocking down osteocyte IFT88, and thus primary cilium, expression. Inhibition of TGF-β receptors, or knockdown via siRNA, blocked cancer cell regulation of osteocyte primary cilia.

DISCUSSION & CONCLUSIONS: These results shed light on a previously unknown mechanism through which osteocyte primary cilia can regulate both breast and prostate cancer cell proliferation. We demonstrate that osteocytes secrete TNF-α which attenuates cancer cell proliferation. However, as the tumour expands, cells release sufficient TGF-β to block osteocyte secretion of TNF-α by disruption of primary cilia. Thus a positive feedback loop exists whereby cancer cells disable the normal osteocyte suppression of cancer cell proliferation thereby enabling further tumour growth.

Our group have recently built an organ-on-a-chip model of metastases, finding that mechanical stimulation of osteocytes regulates both breast and prostate cancer cell behavior[3]. TGF-β mediated disruption of primary cilia by cancer cells, as shown here, may also inhibit osteocyte mechanosensing[4] further impacting bone health and disease. These findings underline the importance of osteocyte primary cilia in regulating bone biology and suppression of breast and prostate metastases.

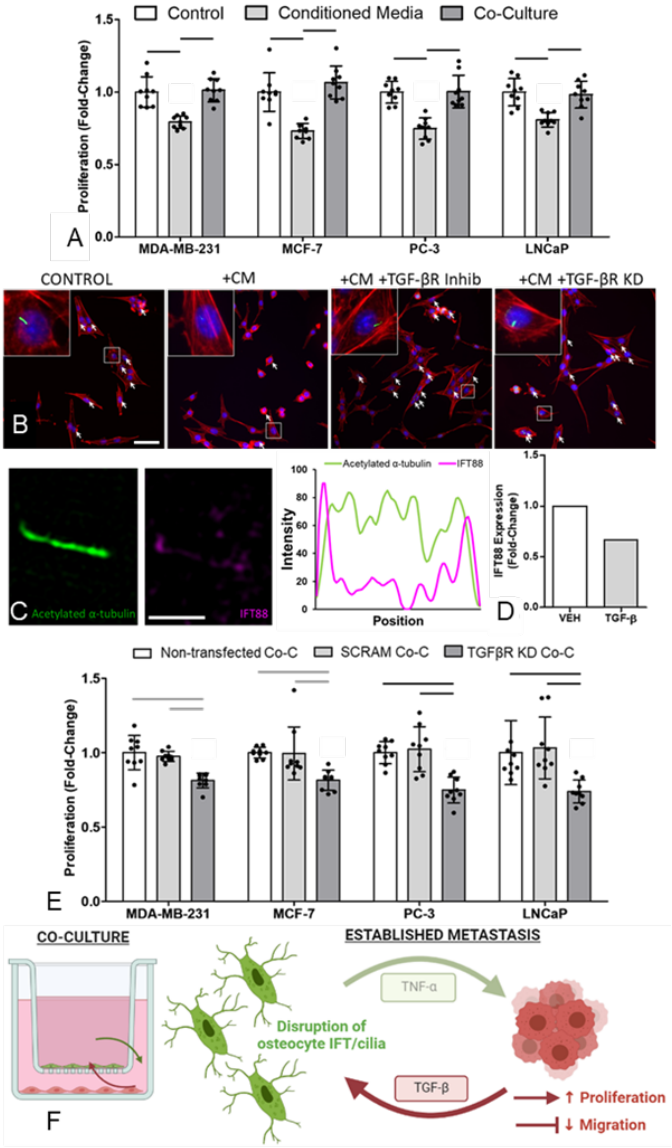

Fig1: (A) Osteocyte conditioned media (CM) reduces cancer cell proliferation, compared to control or co-culture (Co-C). Cancer cells secrete TGF-β in their CM, which (B-D) disrupts osteocyte cilia expression, as measured by confocal, super-res, and qPCR. (E,F) This feedback loop can be blocked to decrease proliferation.

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Multi-organ crosstalk during bone fracture healing - interplay between brain trauma, bone and the immune system

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INTRODUCTION: Despite the remarkably high regeneration capacity of the skeletal system as well as ongoing improvement in fracture treatment, orthopaedic complications such as delayed fracture healing or non-unions are still challenging. It has been shown that the healing process of bone is strongly dependent on blood supply, fracture fixation and other biomechanical factors. However, in recent years the awareness increases that also other factors like age, sex and comorbidities of the patient as well as additional traumata influence bone healing and have to be considered. It has been shown that severe trauma like an additional thoracic trauma, a hemorrhagic shock or even mental trauma delays bone regeneration in preclinical models (1). On the other hand, both clinical and pre-clinical data suggest that an additional traumatic brain injury (TBI) might lead to accelerated fracture (Fx) healing and better mineralized callus, although clinical data are not consistent (2). These findings indicate that there is a multi-organ crosstalk during injury response and an important interplay between the brain, bone and the immune system. The aim of this study was to analyze the presence of inflammatory mediators and immune cells in the circulation and locally in the fracture hematoma and brain early after fracture (Fx) or combined trauma (Fx+TBI) in a mouse model.

METHODS: 24 male C57BL/6J mice were included in the present study at the age of 10-12 weeks. Half of the mice received a unilateral tibia fracture, and the other half received an unilateral tibia fracture and an ipsilateral traumatic brain injury. 6 mice per group were euthanized at 6h after injury and 24h after injury, respectively. Blood was collected and tibiae and brains were embedded into paraffin for further analysis.

RESULTS: To analyze systemic inflammation, several pro- and anti-inflammatory mediators known to be involved in fracture healing were determined in plasma samples at 6h and 24h after injury. G-CSF, IL-6 and IL-10 levels did not differ between Fx and Fx+TBI mice at all time points. KC was significantly increased in the combined trauma group at 6h, but not at 24h after injury. MCP1 was significantly increased in the combined trauma group at 24h, but not at 6h after injury. CXCL10 was significantly reduced in the Fx+TBI mice at both time points. Also locally in the fracture hematoma, CXCL10 gene and protein expression was significantly reduced at both time points. Regarding immune cell populations, significantly fewer neutrophils and mast cells were found in the hematoma of Fx + TBI mice at both 6h and 24h after injury, which correlated with reduced CXCL10 expression. In contrast, significantly more mast cells were found in the brains of Fx+TBI mice.

DISCUSSION & CONCLUSIONS: We found a dysregulated systemic inflammatory reaction in the combined trauma group with the pro-inflammatory cytokines KC and MCP1 being significantly increased, while CXCL10 was significantly decreased. Because we also found less mast cells and mast cells express high amounts of CXCL10, we conclude that less mast-cell derived CXCL10, which is a known pro-osteoclastogenic factor, might contribute to better fracture healing after TBI. We found that mast cell seems to be more likely to migrate to the brain after TBI and therefore are less present in the bone, which indicates an important multi-organ crosstalk between brain and bone, coupled by the immune system.

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Utilizing single cell RNA sequencing to study poor fracture healing

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The failure to produce a robust fracture callus can lead to poor/delayed healing and nonunion. Our laboratory is focused on understanding the cellular and molecular underpinnings that impact callus development and differentiation. Our prior work has utilized a variety of mouse models to study poorly healing fractures; including, genetic, surgical, metabolic and aged models. Herein we have used single cell RNA sequencing (scRNAseq), flow cytometry, and in vitro assays to assess the impact of the genetic deletion of CD47 and ischemia on poor healing. Both conditions result in calluses of reduced size. However, how cellular composition in the developing callus changes with ischemia and/or CD47 knockout is poorly characterized.[1,2,3] Thus, our work seeks to characterize alterations in cell populations under both of these altered fracture conditions to seek common cellular and molecular signatures that lead to poor healing.

C57Bl/6 WT and CD47-null mice were subjected to tibial fracture and compared to WT mice with an ischemic injury prior to tibia fracture. Calluses were manually harvested at days 4 and 7, and we performed scRNAseq. Seurat and Monocle3 were utilized to identify cellular subsets and differentiation trajectories within both the stromal and immune populations. Mice were EdU labeled and harvested at 4 and 7 days post fracture; FFPE IF was performed to analyze cell proliferation. Flow cytometry for a panel of hematopoetic and stromal cell markers was used to characterize callus cells obtained by enzymatic digestion. *In vitro* experiments examining periosteal and marrow mesenchymal stem cell proliferation and differentiation were conducted.

scRNAseq indicates alterations in inflammatory cell populations at day 4 which are confirmed by flow cytometry in both CD47-null and ischemic fractures. Furthermore, there are reduction in cells of the stromal lineage in the ischemic and CD47-null compared to the WT at day 7. This reduction in stromal cells was further confirmed by flow cytometry and EdU assessment via IF.

Differentiation trajectories of the stromal population depict an aSMA+ stromal progenitor cell that follow a direct route towards a chondrogenic fate in WT mice with intact vasculature; however, under ischemic conditions, the cells follow an alternate trajectory through multiple mesenchymal intermediates. Dysregulation in the CD47-null mice is so pronounced that a trajectory from aSMA+ cells to chondrocytes/osteoblasts could not be determined. Gene expression along this trajectory reveals delayed differentiation responses under ischemic conditions. Gene ontology analysis of the stromal population in intact vs CD47-null and ischemic day 7 fracture calluses reveals that terms related to chondrogenesis and osteoblastogenesis are downregulated under ischemic and CD47-null conditions.

Our findings reveal reduced differentiation under both ischemia and CD47 deletion. As CD47-null fractures have enhanced vascularization, proper vascularization does not appear to be a unifying concept.

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Individualized omics-based preclinical models for bone healing

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INTRODUCTION: Bone healing is a spatially controlled process involving crosstalk of multiple tissues [1]. Despite the major advances in osteosynthesis after trauma, there remains a small proportion of patients (<10%) who exhibit delayed healing and/or eventual progression to non-union [2]. While known risk factors exist, e.g. advanced age or diabetes, the exact molecular mechanism underlying the impaired healing is largely unknown and identifying which specific patient will develop healing complications is still not possible in clinical practice. Novel individualized and multimodal omics-based preclinical models for bone healing can provide a platform to precisely address clinically relevant questions.

METHODS: Emerging omics-based methods (e.g. spatial transcriptomics, proteomics) for local and systemic capturing of molecular features during bone healing were assessed for integration into our established *in vivo* femur defect loading set-up in mice [3] consisting of (1) femur defect loading device, (2) time-lapsed *in vivo* micro-CT imaging, (3) 2D-3D registration of histology and micro-CT and (4) endpoint immunohistochemistry [4]. To establish spatial transcriptomics for musculoskeletal tissue from mice, samples were subjected to different pre-treatment protocols and embedded in paraffin. RNA quality was assessed (TapeStation 2200, Agilent) and selected formalin-fixed paraffin-embedded (FFPE) samples were processed on spatial transcriptomics slides (Visium, 10x Genomics) [4]. For *in vivo* longitudinal systemic proteomics analyses, plasma and serum samples were applied to mass spectrometry. Additionally, to non-invasively monitor healing progression *in vivo*, longitudinal stiffness measurements of the fracture repair tissue were performed via a new displacement-controlled loading mode [5] recently implemented into our well-established femur defect loading set-up in mice [3].

RESULTS: Firstly, pre-treatment protocols developed for spatial transcriptomics of FFPE musculoskeletal tissue sections from mice will be presented. The protocols enabled H&E staining of sections on spatial slides successfully meeting the QC requirements for downstream analysis. The obtained gene expression clusters were indicative of specific tissues as seen by spatial overlay with histology. Further, the importance of spatially resolved gene expression data for understanding tissue crosstalk during bone healing will be highlighted. Additionally, first results from proteomics approaches will be presented with a focus on systemic biomarkers associated with impaired healing conditions. In an effort to non-invasively monitor healing over time, a displacement-controlled loading mode was integrated into our femur defect set-up in mice enabling for longitudinal *in vivo* stiffness measurements of the fracture repair tissue and an estimation of the mechanical competence of healing tissue in individual animals.

DISCUSSION & CONCLUSION: Via combining emerging omics technologies for local and systemic biomarker assessment with non-invasive stiffness measurements to assess healing progress we provide an individualized and multimodal platform in mice to precisely address clinically relevant questions on the molecular regulation of bone healing and treatment options.

ACKNOWLEDGEMENTS: Funding from the AO Foundation (AOTRAUMA Network), the European Research Council, the Swiss National Science Foundation and ETH Zurich is acknowledged.

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Release of stem cell paracrine factors, and bone regrowth in patients, induced by composite scaffold

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INTRODUCTION: Functional and aesthetic face rehabilitation has become an important sector of maxillo-facial surgery [1]. Extensive studies also reported a cross-talk between immune and skeletal systems, by which several cytokines and chemokines regulate bone metabolism [2]. In bone tissue engineering, inflammation is an important factor that should be considered to develop successful biomaterial-based therapeutics [3].

METHODS: In this study, the effects of a Hydroxylapatite-Collagen composite scaffold were evaluated using human mesenchymal stem cells (hMSCs), as *in vitro* model. Biomaterial structure was analysed by scanning electron microscopy (SEM). The supernatants were analysed through Bio-Plex Pro Human Cytokine 27-Plex Immunoassay in order to evaluate the expression of cytokines/chemokines released at day 3 and 7. The composite scaffold was employed in maxillo-facial surgery to mold prosthesis during orthognathic surgery in patients with a flat or inadequate projection or asymmetry of the zygomatic area. Shaping is performed according to surgical needs, depending on the clinical evaluation of the patient.

RESULTS: SEM analysis was performed to investigate the microstructure of the scaffold (Figure 1). Stem cells grown on Hydroxylapatite-Collagen composite produced several cytokines/chemokines, compared to the control. Among these, an increase of anti-inflammatory interleukin 4 (IL-4) has been observed at day 7, as well as Interferon-Inducible Cytokine (IP-10) and Vascular Endothelial Growth Factor A (VEGFA) involved in stem cell recruitment and angiogenesis, respectively. The bone substitute gave remarkable aesthetic results in patients in terms of naturalness and symmetry.

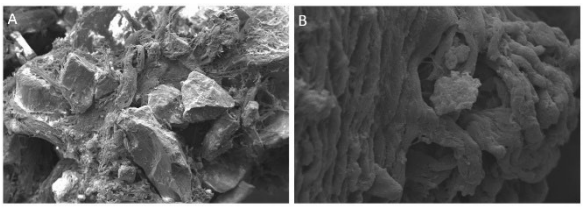

Fig.1: Scanning electron microscopy analysis. Bovine collagen was mixed with Granular Hydroxylapatite to generate the scaffold. Magnification (A) 200X, (B) 4.17 KX.

DISCUSSION & CONCLUSIONS: Our investigation indicates that our model of study can successfully be translated in maxillo-facial surgery for bone repair. Indeed, hMSCs from patients/donors and the composite material could have the advantage of being customized on the basis of the individual patients favouring a personalized medicine approach. Our data demonstrate that the scaffold has immunomodulatory potential and is capable of directing anti-inflammatory innate immune-mediated responses associated to tissue regeneration.

ACKNOWLEDGEMENTS: This work was supported by grants from CARIVERONA Foundation 2020 to AD and MT, PRIN 2017 to FM, and FAR 2023 to EM.

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Transient TGF-β exposure activates BMP-dependent periosteal osteogenesis

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INTRODUCTION: Periosteal osteoprogenitors are the major cellular contributors to appositional bone growth and bone repair by callus formation. Previous work showed that periosteal-derived cells have little or no osteogenic activity under standard in vitro osteogenic culture conditions. This study was conducted to determine what growth factor(s) can activate periosteal osteogenic capacity.

METHODS: RNA was isolated from periosteum from four rapidly growing foals (one month of age) and four adult horses and was analysed by RNA-seq. Subsequently, isolated periosteal cells were maintained in control medium or were exposed to 10 ng/ml TGF-β3 (or 100 ng BMP-2/ml) for 72 hours, then transferred to osteogenic medium.
Changes in osteogenic gene expression (Runx2, OSX and ALP) were measured by qPCR. Osteogenic status was assessed by Alizarin Red staining for mineralized matrix, ALP enzymatic activity and induction of osteogenic genes. To determine whether TGF-β-stimulated osteogenesis required intrinsic BMP activity, TGF-β-pre-treated osteogenic periosteal cultures were co-treated with Noggin or BMP receptor kinase inhibitors, DMH1 and K02288.

RESULTS: As expected, periosteum from foals exhibited transcript profiles reflecting substantially greater osteogenic, matrix synthetic and proliferative activities than adult samples. Surprisingly, there were no significant differences in expression of osteogenic BMP ligands, receptors or intracellular signalling intermediates while two BMP inhibitors, Noggin and BAMBI, were significantly upregulated in foal periosteum. Both TGF-β 2 and 3 ligands, and TGFBR1, were over-expressed in foal periosteal samples.
In vitro, cells initially maintained in control medium exhibited minimal osteogenic differentiation after transfer to osteogenic medium. Pre-treating periosteal cells with TGF-β3 for 72 hours stimulated rapid cell proliferation, ALP up-regulation (Fig 1A), aggregation and mineralization (Fig 1B) in osteogenic medium; in some case, within three days of culture. Pre-treatment with BMP-2 was significantly less effective in inducing periosteal osteogenesis.

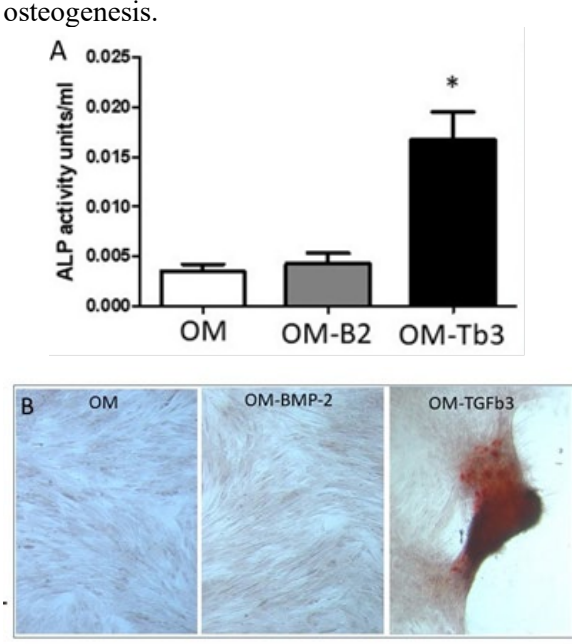

Fig. 1: Periosteal cells initially maintained in control medium (OM) or pre-treated with BMP-2 (OM-BMP2) showed little or no ALP induction (A) or matrix mineralization (B). pre-treatment with TGF-β3 stimulated both responses after transfer to osteogenic medium.

Co-administration of recombinant Noggin or BMP receptor kinase inhibitors suppressed aggregate formation, matrix mineralization, and ALP induction. OSX expression was consistently suppressed, although Runx 2 expression was less consistently affected by BMP signalling inhibition.

DISCUSSION & CONCLUSIONS: Our RNA-seq results indicate that TGF-β signalling is critical for periosteal osteogenesis. Based on in vitro responses, TGF-β is a potent stimulus for periosteal bone formation, although some level of intrinsic BMP activity is necessary. These findings support the development of TGF-β as a therapeutic stimulus for bone repair.

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Longitudinal monitoring of healing progression via in vivo stiffness measurements in a mouse femur defect model.

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INTRODUCTION: Mouse models are frequently used to investigate bone healing. The gold standard in longitudinally assessing fracture healing progression in small animal models is micro-computed tomography (µCT); however, it requires long anaesthesia, causes exposure to radiation, and does not provide a direct assessment of the mechanical competence of the healing tissue. The secondary bone healing process with callus formation is characterised by a progressive increase of fracture stiffness as recently established in a large animal model [1]. The aim of this study was to investigate the feasibility of longitudinal *in vivo* stiffness measurements of the repair tissue in a small animal fracture healing model [2].

METHODS: We extended the capabilities of the previously established mouse femur loading device [2] to enable the *in vivo* stiffness measurements. Following the approval of the local ethics committee (TVB_22/2022, Canton of Grisons, CH) two female C57BL/6J mice received an osteotomy/defect (Table 1) of the right femur stabilised with an external loading fixator (RISystem, Landquart) using established protocols for analgesia and anaesthesia [2].

Table 1. Size of femur defect for each mouse.

	Gap size [µm]
Mouse 1 (small gap)	380um
Mouse 2 (large gap)	920um

Healing progression was monitored by stiffness measurements and µCT (vivaCT 80, SCANCO Medical) conducted directly post-op and then on days 3, 7, 10, 14, 22 and 27. The stiffness was evaluated based on the linear fitting on the force-displacement curve.

RESULTS: Both animals recovered without complications from the surgery and tolerated the stiffness measurements well. For the first ten days post-op the *in vivo* stiffness remained stable at a low level for both animals (Fig. 1). At Day 14, measurements revealed a fourfold increase in stiffness in comparison to the Day 10 measurement for the animal with the small gap. However, the stiffness in the case of the animal with the large gap remained low. µCT conducted at Day 14 showed a robust fracture consolidation in the animal with a small gap and a layer of non-calcified tissue in the defect of the animal with the large gap (Fig. 2). At Day 27, µCT revealed that both fractures were bridged, which corresponded to high *in vivo* fracture stiffness.

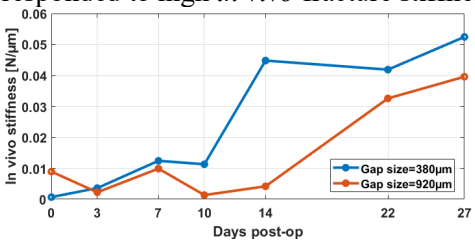

Fig. 1: Stiffness measurements for both animals.

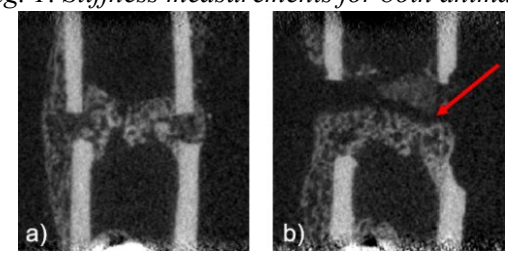

Fig. 2: µCT at Day 14: a) small gap, b) large gap; arrow indicates non-calcified tissue layer.

DISCUSSION & CONCLUSIONS: We demonstrated for the first time that the healing process in the mouse femur defect model can be captured using the *in vivo* stiffness measurements of the fracture repair tissue. When validated on a larger cohort, this methodology could facilitate future studies investigating bone healing in small animals by providing direct estimation of the mechanical competence of healing tissue.

ACKNOWLEDGEMENTS: This study was performed with the assistance of the AO Foundation via the AOTRAUMA Network (Grant No.: AR2022_06). We thank Andrea Furter for performing *in vivo* µCT scans.

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miRNAs Play a Pivotal Role in Fracture Healing

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INTRODUCTION: Immediately after a fracture occurs, a fracture hematoma is formed. This fracture hematoma plays an important role in fracture healing and, under normal circumstances, aids in generating an environment in which a wide variety of cells orchestrate processes involved in fracture healing. In this study, we focus on microRNAs (miRNAs) as they may influence these fracture healing processes. The aim of this study was to determine the miRNA expression signature of human fracture hematoma under circumstances of normal fracture healing and examine the potential influence of clinical parameters on these expression levels.

METHODS: The study was approved by the local ethical committee. Patients with a long bone fracture were screened for inclusion in the study. Harvesting of the fracture hematoma was performed during ORIF fracture surgery. Fracture hematoma was harvested from 61 patients (mean age 52±19; 32♀). miRNA were isolated. Part of each miRNA sample was used to be pooled for qPCR array analysis. Fibrosis- and inflammation qPCR arrays were used. The identified miRNAs were subsequently separately validated in each sample.

Patient characteristics and the time interval between trauma and primary surgery were recorded. Regression analysis was performed.

RESULTS: A total of 145 miRNAs were analysed. In the inflammatory response and autoimmunity array, 76 miRNAs were detected in the fracture hematoma samples, out of which 43 were up- or down-regulated. In the fibrosis array, 80 miRNAs were detected in the fracture hematoma samples, out of which 56 were considered up- or down-regulated. The array data revealed a tendency towards an anti-inflammatory miRNA signature in fracture hematoma and showed a propensity for cytokine activity due to the downregulation of microRNAs which play a central role in cytokine metabolism and regulation. Pro-fibrotic microRNAs were strongly downregulated as compared to anti-fibrotic microRNAs, which showed relatively equal numbers of over- and under-expressed microRNAs. Angiogenic microRNAs were equally over- and under-expressed. Innate- and adaptive immunity microRNAs showed a minimal number of over- and under-expression.

The twenty most regulated miRNAs, 10 up- and 10 down-regulated, were validated. The expression levels of seven out of these twenty miRNAs correlated to several clinical parameters. The time interval between trauma and surgery showed to influence the expression of three miRNAs, three other miRNAs were expressed in a patient age dependent manner and one miRNA was associated with the severity of trauma.

DISCUSSION & CONCLUSIONS: This study portrayed the role and importance of miRNAs in human fracture hematoma, linked to key processes in fracture healing. Seven miRNAs showed to be involved in multiple processes that are important in the fracture healing cascade, such as angiogenesis, mineralization and cellular differentiation. In silico target analysis revealed 124 mRNA targets for 14 out of the 20 validated miRNAs. This study is amongst the first to investigate the expression of miRNAs in patients on this scale and instigates to further investigate the pathophysiology of impaired fracture healing. Moreover, to accurately map potential tissue specific microRNA expression, it is important to research their expression in other tissues related to fracture healing, such as bone itself, periosteum, or soft tissues surrounding a fracture site. Finally, we believe that the in vitro application of microRNAs in both 2D, as well as 3D models, is required to gain more insights into their involvement in biological pathways.

cmRNA and its delivery for Bone regeneration

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INTRODUCTION: Gene therapy was first suggested to treat genetic diseases. More recently, we have come to acknowledge the potentialities of gene therapy in other fields such as regenerative medicine. In this case, gene transfer could be used to deliver gene products that stimulate the regeneration of damaged tissues, including bone. In the mid-1990s, the first applications of gene transfer for bone healing were reported. Since then, a growing interest in the use of gene transfer to deliver osteoinductive growth factors to bone defects has been realized, with bone morphogenetic proteins (BMPs) being the most prevalent growth factor of choice. Although considerable progress has been made, applications in bone healing remain at the preclinical research stage. Few constraints have been identified that limit the translation of gene therapy to bone healing in patients. Issues related to safety, inefficient gene transfer, immunogenicity, and affordability are among the most important limitations of gene therapy translation. While many investigations have been conducted to develop safer gene therapy vectors, affordability remains to be a pressing issue with gene therapy products in the market tagging € 1 Mio per treatment.

A new type of gene therapy, often called transcript therapy, has been recently described. By means of transcript therapy, messenger RNA (mRNA) is used for gene transfer instead of the commonly employed plasmid DNA (pDNA). mRNA offers numerous advantages over pDNA. mRNA does not require transport across the nuclear membrane, it typically acts effectively upon release in the cytosol. It is functional in dividing and non-dividing cells, and, unlike pDNA, it does not hold risks associated to genome integration. Furthermore, mRNA production is simpler and more affordable than pDNA. Therefore, it promises to expedite the translation of gene therapy in regenerative medicine.

This talk seeks to introduce the audience to transcript therapy concepts, their advantages, and their limitations. The state-of-the-art of transcript therapy for bone regeneration will be presented. Several examples of our own research will be provided to illustrate the uses of protein coding mRNA for bone healing. Results from *in vitro* evaluations using different primary cell types and tissue explants will be shown. Relevant aspects when considering mRNA transfer for tissue regeneration, such as ideal vectors and 3D scaffolding materials, will be emphasized. In addition, evidence on the safety and efficacy of this technology gained in rodent models of bone healing will be given. Future perspectives will be highlighted.

Intelligent CRISPR Design for Bone Regeneration

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Gene editing by CRISPR and gene regulation by microRNA or CRISPR activation have dramatically changed the way to manipulate cellular gene expression and cell fate. In recent years, various gene editing and gene manipulation technologies have been applied to control stem cell differentiation to enhance tissue regeneration. This presentation will focus on how to develop CRISPR, CRISPR activation (CRISPRa), CRISPR inhibition (CRISPRi) as well as bi-directional CRISPR-AI gene regulation technologies to control cell differentiation and bone regeneration.

Vector Engineering and Development for Osseous Nonunions

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INTRODUCTION: Long bone fractures usually heal spontaneously, but approximately 100,000 fractures in the USA each year fail to do so, resulting in non-unions that can cause significant morbidity. Large, critical-size, segmental defects never heal spontaneously and are particularly troublesome. Current treatments for such defects include the osteogenic protein recombinant human Bone Morphogenetic Protein-2 (rhBMP-2) delivered via a collagen sponge. While rhBMP2 promotes bone healing, its clinical potency is poor, necessitating the use of high doses that cause detrimental side effects, and generate poor quality bone. Prior work from this laboratory suggests that genetic transfer of BMP-2 is superior to delivery of rhBMP-2 for bone healing. Recently we have shown that delivery of cDNA encoding interleukin-1 receptor antagonist (IL-1Ra) increases the potency of BMP-2 [1]. Based on these findings, we are engineering and developing novel vectors for delivering genes to osseous defects with a focus on Adeno-associated virus (AAV).

METHODS: AAV vectors of several different serotypes encoding a GFP-luciferase fusion protein were tested on rat bone marrow mesenchymal stromal cells to determine which variety possessed a natural tropism for the likely target cell type within an osseous defect (Figure 1). This identified AAV2.5 as the most potent serotype.. Using this serotype, a monocistronic vector encoding BMP-2 (AAV2.5-BMP2) and a bicistronic vector encoding both BMP-2 and IL-1Ra (AAV2.5-BMP2-IL1RN) were constructed (Figure 2). In a pilot study, $1x10^{10}$vg of each vector was loaded onto a collagen sponge and implanted into 5 mm, critical sized, diaphyseal, rat femoral defects [1].

RESULTS: All vectors were successfully constructed, produced and purified for 100ul doses of $1x10^{10}$vg. By 12 weeks post-surgery little or no osteogenesis was noted in defects receiving AAV2.5-BMP2. Implantation ofAAV2.5-BMP2-IL1RN led to the deposition of considerable amounts of new bone (Figure 3).

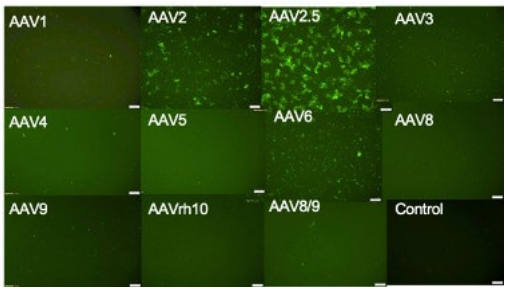

Fig. 1: Transducing ability of AAV Serotypes in Rat Bone Marrow Mesenchymal Stromal Cells

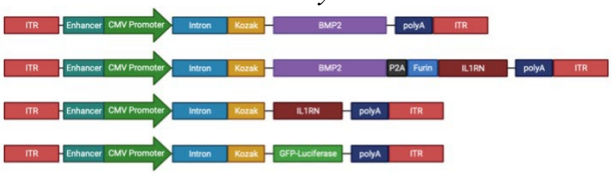

Fig. 2: AAV Construct Design

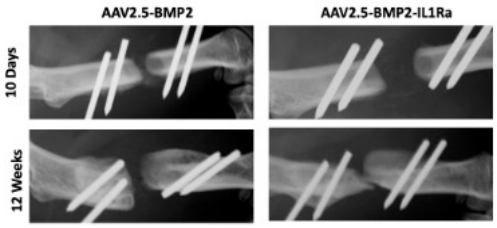

Fig. 3: AAV2.5 with and without the addition of IL1RN in a critical-sized defect in male rats.

DISCUSSION & CONCLUSIONS: These preliminary data identify a bicistronic AVV vector encoding both BMP-2 and IL-1Ra as a promising new reagent for promoting bone formation. Higher doses of the vector should lead to complete bridging, The data further substantiate the results obtained by *Panos et al.* [1] showing enhanced BMP-2 potency when IL-1Ra is expressed locally via gene transfer.

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Improving mineralization in a novel fracture hematoma model via delivery of IL-10 and BMP-7

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INTRODUCTION: In large bone defects, where classical treatments fail to achieve healing, regenerative biomaterials in combination with immunomodulatory therapies show great promise. Increasing evidence suggests that interleukin-10 (IL-10) and bone morphogenetic protein-7 (BMP-7) have potent immunomodulatory properties. However, their role during fracture hematoma formation and subsequent regulation of bone regeneration remains unknown. This is in part due to the limitations in models of fracture healing, which do not take into account the immunoregulatory role of blood and its interaction with biomaterials. The aims of this study were therefore to (i) establish a model of the fracture hematoma to investigate the cross-talk between blood and bone mineralization in a regenerative biomaterial, and (ii) functionalize the biomaterial with IL-10 and BMP-7 to enhance mineralization under inflammatory conditions.

METHODS: Collagen-hydroxyapatite (CHA) scaffolds were incubated with blood and the adsorption of fibrinogen and fibrin network formation was assessed using two-photon excitation microscopy (TPEF). Bone progenitor cells (HBCs) were seeded on CHA scaffolds pre-incubated with blood and the influence of blood on cell migration and mineralization was evaluated using TPEF, µCT reconstructions, ALP and calcium quantification. Cytokine arrays and enzyme linked immunosorbent assays (ELISAs) were used to identify signalling molecules involved in the regulation of mineralization. The effect of blood on the release kinetics of IL-10 and BMP-7 from CHA scaffolds was determined using ELISAs, while the effect of functionalized scaffolds on HBCs was determined using mineralization assays. All experiments were repeated using at least 3 donors of blood and HBCs.

RESULTS & DISCUSSION: Following incubation in blood, fibrin network formation limited the capacity of HBCs to migrate into CHA scaffolds (*Fig. 1A-B*). The mineralization capacity of HBCs was significantly reduced by blood (*Fig. 1C*) in conjunction with an upregulation of leptin, osteopontin, serpin E1, IL-6 and IL-8 signaling. This suggests that blood contributes to the regulation of HBCs differentiation into bone-forming cells via immunoregulatory signaling. Having successfully functionalized CHAs with IL-10 and BMP-7, it was found that blood could stimulate their release and that matrix-metalloproteases were involved in the process. The release of IL-10 and BMP-7 enhanced the mineralization capacity of HBCs, particularly in the presence of blood. Taken together, this suggests that IL-10 and BMP-7 can serve as a co-therapy to stimulate bone regeneration under inflammatory conditions.

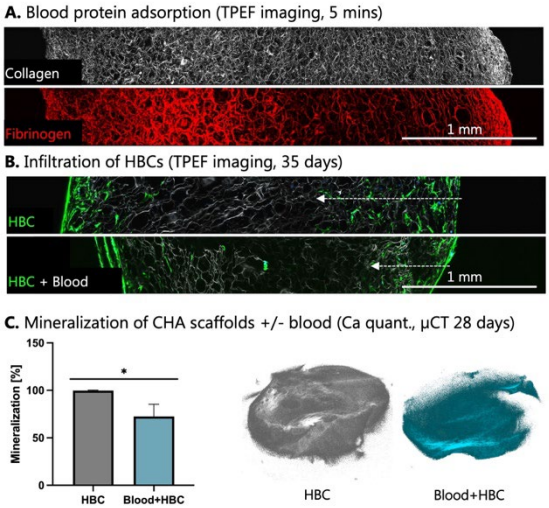

Fig. 1: Cross-section of CHA scaffolds showing (A) the adsorption of blood proteins, and (B) the impairment of HBC infiltration. (C) Ca^{2+} quantification and µCT reconstructions show that blood limits mineralization.

CONCLUSIONS: Notably, this study presents both a new model to investigate how hematoma formation can modulate mineralization and a new immunomodulatory strategy to advance bone regeneration.

ACKNOWLEDGEMENTS: This research was funded by Empa and an ON/ORS Kick-Starter Grant.

Feasibility of Use of a Non-Osteogenic, Genetically Modified Cell Line for Large Bone Regeneration in a Rat Femoral Segmental Defect Model

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INTRODUCTION: Despite bone's remarkable ability to heal, up to 10% of all fractures result in non-unions. Bone morphogenetic protein -2 (BMP-2) is a potent osteoinductive growth factor but has failed to reach wide adoption as a fracture healing adjuvant for trauma. Its use has been limited by high costs and side effects associated with the supraphysiological doses required by the recombinant protein. The use of *ex vivo* gene transfer using autologous mesenchymal stromal cells (MSCs) has been proposed. While successful in pre-clinical models, its clinical application, is limited by the high costs and hurdles associated with harvesting, culture expansion, transduction, and characterization under GMP conditions. This project explores the feasibility of obviating these issues with a stable, genetically modified, cell line to deliver BMP-2 to a rat bone defect.

METHODS: HEK293 cells were transduced with a lentivirus carrying the human BMP-2 cDNA driven by the CMV promoter. Clones were selected by puromycin, expanded, BMP-2 production characterized by ELISA and cells frozen at either $1x10^6$ (low), $3x10^6$ (medium) or $5x10^6$ (high) cells/vial. Individual vials were thawed, and cells encapsulated in fibrin just prior to surgical implantation in a rat, 5-mm, femoral, segmental bone defect model (n=10/group). Non-transduced HEK293 cells and fLuc expressing HEK293 cells were used as controls (n=5/group). FK506 was used to prevent xenograft rejection. Defect bridging was monitored via radiographs and rats were euthanized at 12 weeks. Bone samples were scanned by microCT, processed for histology analysis and/or biomechanical testing.

RESULTS: One clone (CL1K) was selected, and BMP-2 production characterized for 50 passages (Fig. 1A). Bridging of the defect appeared to be correlated with the number of implanted CL1K cells, with the low, medium, and high cell dose achieving 60%, 70% and 80%, respectively. Bridging variability was evident in all defects receiving CL1K cells (Fig. 1B). MicroCT analysis showed no statistical difference in values of BV, TV, and BV/TV between CL1K treated defects, nor with contralateral femur controls. Torsional testing of the bridged femora showed no difference between CL1K treated defects, although the middle and high dose groups were statistically weaker than femur controls ($p<0.05$), but not the low dose group ($p=0.159$). Histological analysis showed evidence of abundant new bone formation in the bridged defects, with areas of endochondral ossification, while defects that failed to bridge had fibrous tissue mixed with areas of new bone formation within the defect. Unmodified HEK293 and fLuc expressing cells showed no evidence of bone healing.

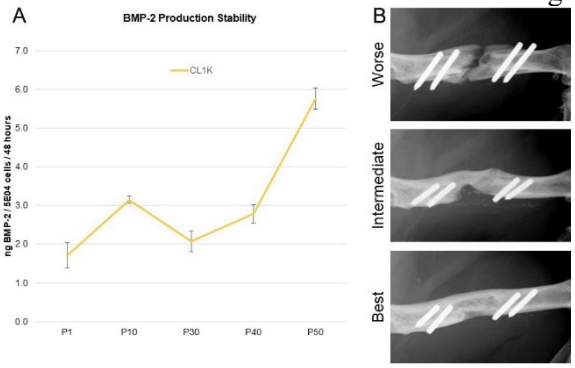

Figure 1. (A) BMP-2 production by CL1K cells after 50 passages. (B) Defect bridging variability of implanted CL1K cells as evidenced by radiographs.

DISCUSSION & CONCLUSIONS: HEK293 cells do not differentiate into osteoblasts, yet these data show that BMP-2 expressing HEK293 cells stimulate healing of a large bone defect. Traditional cell and gene therapy approaches to bone regeneration have used MSCs, we here demonstrate the feasibility of using non-osteogenic cell lines for this purpose. While HEK293 cells are not ideal for clinical translation, this project serves as proof of concept for a novel approach that might facilitate the use of cell therapies as bone healing adjuvants.

ACKNOWLEDGEMENTS: This project was partially funded by the Mayo Clinic Benefactor Funded Grant Award for Musculoskeletal Research.

Demonstrating microbubble perfusion and cavitation within a murine critical calvarial defect.

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INTRODUCTION: Bone fractures are common injuries with an individual lifetime risk of 38%.(1) Approximately 10-15% of these injuries will go on to develop a non union, defined as no radiological evidence of healing at 9 months following injury(2). This is a debilitating condition causing significant pain and disability. The current treatments for this condition are costly and often involve invasive procedures such as revision surgery. There is a paucity of non operative treatments available for this vulnerable patient group. Microbubbles are historically used as contrast agents in ultrasound imaging: they can interact with ultrasound waves and undergo either stable or inertial cavitation. This can cause the bubbles to break up and deposit a payload(3). In addition, cavitation causes vascular endothelium and cell membranes to become more porous leading to greater uptake of local compounds.

METHODS: Male adult MF1 mice underwent calvarial defect surgery during which a 5mm crown drill was used to create a defect in the centre of the skull over the Sagittal suture between the parietal bones. Animals had their tail veins cannulated and were placed on a specially adapted jig under anaesthesia. Peak pressures achieved were approximately 740kPa with a duty cycle of 1%. They were exposed to ultrasound energy for a total of 7 minutes. They had 100ul of SonoVue injected at 2 minutes and the acoustic signal was recorded using TiePie software. The data was processed using MatLab software and the results analysed. Animals also underwent uCT scanning.

RESULTS: Four mice underwent surgery and were subsequently scanned at weekly intervals for 4 weeks giving 16 data points. The defects created underwent minimal bony regeneration as shown with micro CT. The data demonstrated cavitation of microbubbles at all time points following surgery with greater intensities of signal change seen at 2 and 3 weeks (-7.5 and -6.5 dB re W).

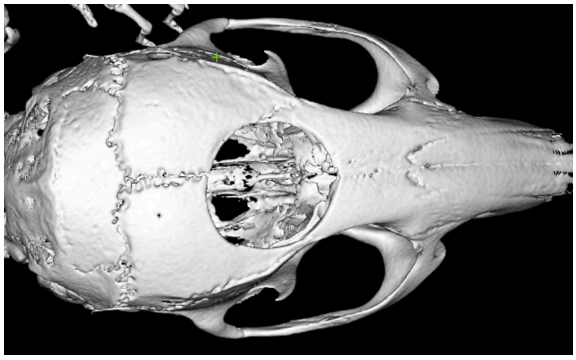

Fig. 1: Calvarial defect surgery demonstrated on micro CT scanning.

DISCUSSION & CONCLUSIONS: The results demonstrate the ability to initiate inertial cavitation within a bony defect using commercially available microbubbles at energy levels that are safe for animals with no evidence of neurological deficit. This represents a viable model for testing the ability of loaded microbubbles to deliver compounds to fracture sites, and a viable model for use with a targeted ultrasound jig.

ACKNOWLEDGEMENTS: This research was funded by Orthopaedic Research UK

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Biodegradable magnesium alloys for trauma implants

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INTRODUCTION: Magnesium (Mg)-based alloys have become an important category of materials that is attracting more and more attention due to their high potential use as orthopedic temporary implants. These alloys are a viable alternative to nondegradable metals implants in orthopedics. Biodegradable metals are a new paradigm for orthopedic implants because they promote bone formation and sustain the bone healing and remodeling process through a gradual load transfer between implant and tissues. In addition, they are designed to provide sufficient mechanical strength at the beginning of the treatment, and then, after their degradation, a complete bone healing process is observed.

METHODS: In this paper, a detailed overview covering alloy development and manufacturing techniques is described. The key features for biodegradable Mg alloys, suitable for temporary orthopedic implants, are biocompatibility, proper mechanical properties to assure mechanical integrity until the fracture healing, degradation rate, and dynamic corrosion, according to the clinical needs. The effective biofunctionality of the biodegradable temporary orthopedic implants can be evaluated only by in vivo testing, on animal models, followed by clinical trials. In addition, the new bone formation, bone–implant interface, and inflammatory reactions can be evaluated. The methods used for the evaluation of the biodegradable Mg alloys for temporary orthopedic implants are shown in Figure 1.
Special attention is given to animal testing, and the clinical translation is also reviewed, focusing on the main clinical cases that were conducted under human use approval.

RESULTS: Different research groups demonstrated that the initial difficulties with in vitro testing appear to be surpassed, and the testing procedures and the mediums used are quite well accepted. Animal testing is widely presented in the literature, and the main species used for in vivo experiments are mice, rats, rabbits, dogs, goats, and mini pigs. The advantages and drawbacks of these animal models were highlighted, and they can be correlated also with the in vitro tests.

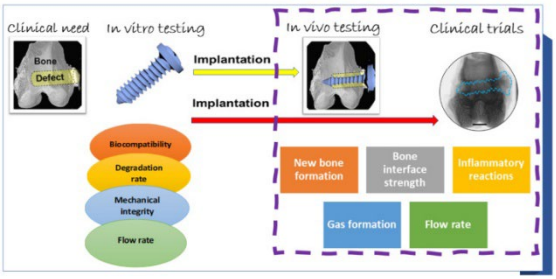

Fig. 1: Methods used for the evaluation of the biodegradable Mg alloys for temporary orthopedic implants [1]

DISCUSSION & CONCLUSIONS: The objective of this paper was to present a systematic investigation on different Mg alloys from different system. Future research must be concentrated on the direction of alloys with a low degradation rate and an improved mechanical strength, in order to solve load-bearing zone fractures. New designs for orthopedic implants are possible to be developed in the near future, especially for foot and ankle surgery, if the researchers better correlate the clinical needs with each Mg-based alloy biofunctional properties. This is because, in the case of biodegradable Mg-based alloys, we cannot conclude that a universally accepted alloy for any orthopedic applications exists. Further research that includes human studies is indicated for each newly developed implant.

ACKNOWLEDGEMENTS: -
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3D-printed bone substitutes for osteoconduction and bone augmentation: When architecture meets biology.

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INTRODUCTION: In the last decades, advances in bone tissue engineering mainly based on osteoinduction and on stem cell research. Only recently, new efforts focused on the micro- and nanoarchitecture of bone substitutes to improve and accelerate bone regeneration. By the use of additive manufacturing, diverse microarchitectures were tested to identify the ideal pore size [1], the ideal filament distance and diameter [2], or light-weight microarchitecture [3], for osteoconduction to minimize the chance for the development of non-unions. Overall, the optimal microarchitecture doubled the efficiency of scaffold-based bone regeneration without the need for growth factors or cells. Another focus is on bone augmentation, a procedure mainly used in the dental field. Presently, we test triply periodic minimal surface microarchitectures which combine light-weight and high strength and look at 10 days of defect healing to study early events leading to osteoconduction.

METHODS: For the production of scaffolds, we applied the CeraFab 7500 from Lithoz, a lithography-based additive manufacturing machine. Hydroxyapatite-based and tri-calcium-phosphate-based scaffolds were produced with Lithoz TCP 300 or HA 400 slurries. The evaluation of triply periodic minimal surface (TPMS) microarchitectures was performed with diamond, gyroid, primitive in comparison to a lattice microarchitecture, all with a minimal percolation of 0.8 mm in diameter. As in vivo test model, we used a calvarial defect and a bone augmentation model in rabbits. 1-sided-bone ingrowth into the defect and the ingrowth into the bone augmentation model was determined based on the middle sections of toluidine-stained ground sections. In vitro tests were performed with osteoclasts and bone marrow derived mesenchymal stem cells. Gene analysis of a high and low osteoconductive scaffold from the identical material, porosity, microporosity and transparency were compared to identify microarchitecture specific genes leading to osteoconduction.

RESULTS: The histomorphometric analysis revealed that bone ingrowth was significantly increased with pores between 0.7-1.2 mm in diameter. Best pore-size for bone augmentation was 1.7 mm in diameter. Therefore, pore-based microarchitectures for osteoconduction and bone augmentation are different. For TPMS-based microarchitectures, we saw gyroid significantly better than primitiv in both osteoconduction and bone augmentation. Moreover, microporosity appeared to be a strong driver of osteoconduction and influenced osteoclastic degradation for tri-calcium phosphate-based scaffolds. For hydroxyapatite-based scaffolds, however, microporosity appears to influence osteoconductivity to a lesser extent. Osteoclasts were able to degrade hydroxyapatite-based scaffolds irrespective of nanoarchitecture but tri-calcium phosphate-based scaffolds only at high and moderate levels of microporosity. The evaluation of the gene expression profiles at early bone healing leading to osteoconduction or preventing it are still ongoing and will be presented at the meeting.

DISCUSSION & CONCLUSIONS: Micro- and nanoarchitectures are key driving forces for osteoconduction and bone augmentation. Triply periodic minimal surface microarchitectures can be realized by additive manufacturing. Since they combine light-weight with high strength, they appear as the ideal microarchitecture for bone substitutes. We identified the gyroid microarchitecture as superior for bony bridging and bone augmentation purposes. Based on these new results additive manufacturing appears as the tool of choice for the production of personalized bone tissue engineering scaffolds to be used in cranio-maxillofacial surgery, dentistry, and orthopaedics, since TPMS-based scaffolds can't be produced by other means.

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Osteoclastic resorption of calcium phosphate-based materials is influenced by the crystallization pathway

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INTRODUCTION: Osteoclastic resorption of calcium phosphate (CaP) materials is essential for osseointegration and remodeling *in vivo*. Osteoclasts (OCs) also stimulate osteoblast activity. While OC differentiation and resorption on CaP surfaces is influenced by chemical composition, Ca/P molar ratio, crystallinity, and topography, the effect of different amorphous calcium phosphate (ACP) crystallization pathways on OC resorption is unclear. The aim of the study was to compare primary OC resorptive activity on surfaces of two CaPs (ACP and crystallized ACP resembling nanohydroxyapatite, nHAp) by evaluating OC properties, gene expression markers, and CaP surface morphology changes up to 10 days.

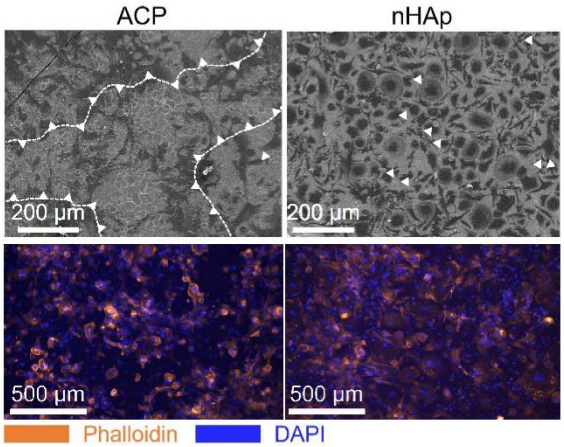

Fig. 1: Representative SEM images (top) of the osteoclast resorbed pit areas (ACP) and individual spots (nHAp) and EPF images (bottom) of DAPI and Phalloidin stained osteoclasts on ACP and nHAp-like materials at day 10.

METHODS: ACP was synthesized through precipitation. Part of it was matured in water, thus obtaining nHAp-like CaP. The ACP and nHAp-like powders were uniaxially compacted into discs, sterilized, and conditioned in cell culture media before *in vitro* tests. Conditioning of pure ACP also crystallized it. Mononuclear cells were retrieved from the bone marrow of two human donors with ethical consent and differentiated into pre-OCs. The pre-OCs were seeded on ACP and nHAp-like discs ($6x10^5$ cells/cm^2) and cultured in complete culture media containing M-CSF and RANKL for 7 and 10 d. Control OCs were seeded on plastic. Cells were characterized using epifluorescence microscopy (EPF) for DAPI (nuclei) and Phalloidin (actin ring) staining. The expression of OC differentiation markers was analysed by quantitative polymerase chain reaction (qPCR). Scanning electron microscopy (SEM) was used to evaluate the number of OC resorption pits.

RESULTS: Phalloidin and DAPI staining revealed fully formed actin rings and one or few nuclei per OC, while the control OCs were multinucleated. Expression of the OC markers *ACP5*, *MMP9*, and *CA2* was upregulated for both materials in comparison to positive control at day 10. The number of resorption pits was higher for ACP than for nHAp. Large areas of ACP surface were covered in resorption pits while only few pits were observed on nHAp (Fig.1).

DISCUSSION & CONCLUSIONS: We compared OC resorptive activity on two CaPs derived from different ACP crystallization pathways: in water or cell culture media. Both CaPs supported the differentiation of pre-OCs into OCs. Both CaPs facilitated similar OC marker expression levels, but distinct resorption patterns (few pits for nHAp vs. pit-covered surface for ACP). This discrepancy could arise from variations in the chemical composition, crystallinity, and solubility of the studied CaPs. By selecting the crystallization pathway, a rather small technological nuance, we can manipulate the extent of resorption (high for ACP or lower for nHAp), tailoring it to specific bone regeneration strategies. These findings can enhance the development of more effective CaP-based biomaterials for f-uture orthopaedic applications.

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3D Printed Composite Resembling Natural Bone by Combining Hyaluronan, Collagen and Calcium Phosphate to Promote Bone Regeneration

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INTRODUCTION: Large bone defects present a major clinical challenge due to insufficient self-healing. The gold standard, autografting, has serious limitations like donor site morbidity. Alternatives are engineered bone graft substitutes, however current available options lack spatial control over scaffold architecture to anatomically match complicated bone defects. This study aims to develop a composite biomaterial-ink of tyramine modified hyaluronic acid and collagen type I (THA-Col) mixed with osteoinductive calcium phosphate particles (CaP), that is 3D printed to fabricate patient-specific bone graft substitutes.

METHODS: The composite comprises 35 mg/mL THA combined with 5 mg/mL rat tail Col mixed 1:1, supplemented with 0.5 U/mL horseradish peroxidase (HRP) and 0.01% w/v Eosin Y mixed with a range of 0-30% w/v CaP (size 45-63 or 45-106 µm). Upon adding 0.085 mM H_2O_2 and 6 mM NaOH a 3D printable gel is formed. Post 3D printing, matrices were fully cured using photo crosslinking (505 nm). Matrices were characterized by rheology, swelling, and compressive modulus. Further, composites were assessed *in vitro* for cytotoxicity using human mesenchymal stromal cells (hMSCs) seeded on the matrices and analyzing viability with live/dead assay for up to 7 days. *In vitro* osteogenic potential of composites with hMSCs was analyzed by alkaline phosphatase (ALP) production after 14 days, gene expression analysis (*RUNX2, SOX9, ALPL, IBSP, SPP1, MMP13, COL1A1*) at 14 and 28 days and osteoprotegerin (OPG) production over 28 days of differentiation.

RESULTS & DISCUSSION: Rheology revealed the viscoelastic and shear-thinning behavior of THA-Col, desirable for 3D printing. The inclusion of CaP reduced swelling of THA-Col, which is advantageous for preserving 3D printed structures. THA-Col compressive modulus showed an increase with increasing CaP content. The *in vitro* analysis of hMSCs on composites, showed high viability on all composites on day 7 (**Fig. 1A**). *In vitro* osteogenic differentiation resulted in a more intense ALP staining on THA-Col + 0% CaP followed by THA-Col + 10% CaP (**Fig. 1B**), which was also confirmed by ALP activity assay (**Fig. 1C**). OPG secretion further validated these results, with highest production in THA-Col (**Fig. 1D**). Gene expression also indicated similar trends.

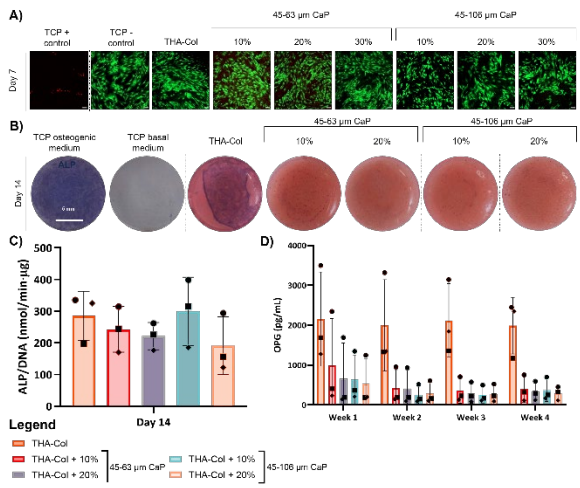

Fig. 1: *A) Live (green) and dead (red) staining of hMSCs at day 7, scalebar = 100 µm. B) ALP staining (blue) after 14 days of hMSCs osteogenic differentiation, scale bar = 5 mm. C) ALP activity normalized to DNA content after 14 days of hMSCs osteogenic differentiation. D) OPG secretion of hMSCs during osteogenic differentiation. ● Donor 1 (82y, f) ■ Donor 2 (61y, f) ♦ Donor 3 (80y, f).*

CONCLUSIONS: Here, a 3D printable composite of THA-Col and different sizes and concentrations of CaP was developed, which holds significant potential to serve as a patient-specific bone graft substitute for the regeneration of large bone defects.

ACKNOWLEDGEMENTS: This work was supported by the EU Horizon 2020 research and innovation program under grant agreement No. 874790.

CHITOSAN-MODIFIED DENSE COLLAGEN HYDROGELS LOADED WITH ANTI-SCLEROSTIN ANTIBODIES AS BIOMATERIALS FOR CRITICAL SIZE CALVARIA DEFECT REPAIR

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INTRODUCTION: A tissue engineering strategy based on dense collagen hydrogels (DCH) is a promising approach to restore bone defects especially in combination with bioactive molecules or mesenchymal stem cells (1). Here, we aimed at developing DCH-derived scaffolds enclosing and delivering anti-Sclerostin antibody. To finely tune the antibody release profile, chitosan, a biocompatible cationic polysaccharide, was added. The addition of dental pulp stem cells (mDPSC) was then evaluated to improve further bone reparation.

METHODS: DCH-chitosan (10% of chitosan, 3 different molecular weights) mixed scaffolds were prepared using the plastic compression process. They were studied by micro-X-ray, computed tomography, rheology and scanning electron microscopy. Antibody release kinetics were studied by ELISA. In vivo (APAFIS agreement # 24,297), a 3.5 mm critical size defect was surgically created in the parietal bone of 10-week-old male WT mice with defects subjected to the following conditions: left empty, DCH +/- chitosan +/- anti-Scl Ab. A similar experiment with addition of mDPSC was realised with the chitosan showing the best results. Bone formation was assessed by Micro-CT at 1 and 2 months and by immuno-chemistry. Statistical analyses were performed using non-parametric tests. Significance was defined as a p-value lower than 0.05.

RESULTS: No structural difference was found between DCH-chitosan and DCH alone. Antibody loading was significantly improved upon chitosan addition ($p<0.05$). Significantly improved bone formation was observed in mice at 1 and 2 months with antibody loaded DHC when compared with no DCH or DCH alone ($p<0.05$). Addition of chitosan, with or without antibody, slowed down bone formation (Fig 1). Adding mDPSC didn't improve further bone formation.

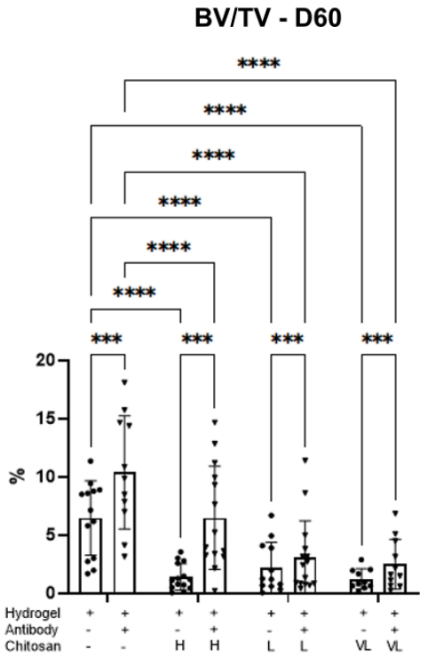

Fig. 1: Effect of antisclerostin antibody and chitosan on the bone formation (Bone volume/Tissue volume) in critical size bone defects at 2 months in mice. H/L/VL: high / low / very-low molecular weight.

DISCUSSION & CONCLUSIONS: Anti-sclerostin antibody loaded collagen-based hydrogels are promising biomaterials to repair critical-size bone defects. Despite their biological properties, chitosan or mDPSC didn't improve in vivo efficiency compared with anti-sclerostin antibody alone.

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In vivo study of 3D-printed polycaprolactone-hydroxyapatite scaffolds with Voronoi design to advance the concept of scaffold-guided bone regeneration

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INTRODUCTION: Scaffold-guided bone regeneration (SGBR) using biodegradable medical-grade polycaprolactone (mPCL) composite scaffolds 3D-printed with rectilinear infill has clinically been proven a valuable concept in the treatment of large bone defects [1]. However, progress in 3D printing technology is now enabling the additive manufacturing of advanced morphologies for next-generation (4.0) design scaffolds, such as those with Voronoi tessellation. We hypothesized that Voronoi designs would allow optimization of mechanical properties while retaining important morphological features from an SGBR perspective, i.e., high porosity and interconnectivity, whereas rectilinear infill scaffolds are known to lack this versatility. To test this hypothesis, we performed an in-depth *in vivo* analysis of generation 4.0 Voronoi scaffolds made of mPCL-hydroxyapatite (mPCL-HA).

METHODS: Tubular 3D-printed mPCL-HA composite (wt 96%:4%) scaffolds with Voronoi design (outer diameter 10 mm, inner diameter 4 mm and a height of 12 mm, porosity 50.44 ± 3.53%), with and without loading with different types of freshly harvested sheep bone graft material were tested *in vivo* in a rat ectopic bone formation model with follow-up of eight weeks.

RESULTS: Macroscopically apparent highly vascularized tissue without extensive fibrous encapsulation was found in all mPCL-HA scaffolds. Immunohistochemical (IHC) analysis of the demineralized samples and scanning electron microscopy (SEM), fluorochrome dye imaging, Goldner trichrome and rhodamine staining of the mineralised samples showed active bone regeneration throughout the Voronoi scaffold architecture, particularly at the surface of the bone chips with endochondral bone formation, while highly vascularized tissue formed in the porous space (Fig. 1). In addition, slow scaffold degradation by SEM, molecular weight changes by gel permeation chromatography (p = 0.27) and crystallinity by differential scanning calorimetry (p = 0.05) were observed.

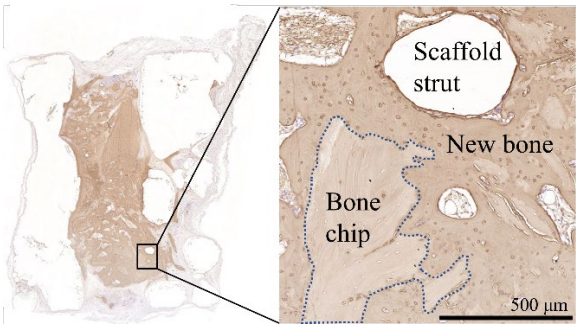

Fig. 1. Exemplary IHC image (collagen type I staining) of an in vivo scaffold loaded with xenogeneic graft material indicating successful bone regeneration throughout the porous architecture of the mPCL-HA Voronoi scaffolds.

DISCUSSION & CONCLUSIONS: To pave the way for the clinical implementation of the next generation of biodegradable scaffolds, we conducted a comprehensive *in vivo* study of a 3D-printed Voronoi scaffold design (generation 4.0 scaffolds) fabricated from mPCL-HA. Good biocompatibility and biointegration of mPCL-HA Voronoi scaffolds, with and without loading with fresh xenogeneic bone graft, along with slow degradation *in vivo* indicate their suitability for subsequent large animal and clinical studies to further advance the SGBR concept.

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Mast cells - Crucial modulators in osteoporotic fracture healing

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Mast cells are tissue-resident immune cells which store a large number of secretory granules which contain a plenty of preformed mediators, including histamine, cytokines, and various growth factors. They are located in various tissues, including the bone marrow and are suitable candidates to be involved in bone metabolism and disorders, as many of their mediators are well-known regulators of osteoblast and osteoclast activities (reviewed in *Ragipoglu et al. 2020*). Indeed, mast cells appear to play a role in the development of osteoporosis, as their number is enhanced in the bone marrow of osteoporotic patients. Furthermore, systemic mastocytosis, a disease characterized by mast cell hyperplasia in various organs, including the bone marrow, often induces bone loss (reviewed in *Ragipoglu et al. 2020*). Confirming these clinical observations, we demonstrated that mast cell-deficient mice (Mcpt-5 Cre R-DTA) were protected from ovariectomy (OVX)-induced bone loss, a murine model for postmenopausal osteoporosis. The strong increase of osteoclast number and activity, which is normally observed in OVX-mice, was completely abolished in mast cell-deficient mice. This suggests that mast cells promote bone resorption under estrogen-deficient conditions (*Kroner et al., 2017*). To unravel the underlying mechanism, we studied osteoclast formation *in vitro* in the presence of mast cell supernatants. Supernatants of mast cells, which were stimulated with the anaphylatoxin C5a to induce degranulation, enhanced osteoclast formation. When mast cells were treated with estrogen, their osteoclastogenic effect was abolished (*Kroner et al., 2017, Fischer et al. 2022*). Further experiments revealed that mast cells regulate osteoclast formation by releasing the osteoclastic mediators Midkine and CXCL10 in an estrogen-dependent manner (*Fischer et al. 2022*).

Mast cells may also play a role in bone healing. Several phenomenological studies described their appearance in the fracture haematoma and in the fracture callus (reviewed in *Ragipoglu et al. 2020*). However, their function in bone healing has been unknown so far. Using again mast cell-deficient mice, we showed that mast cells trigger local and systemic inflammation after fracture by secreting inflammatory mediators, including IL-6, IL-1ß, and TNF-α and by inducing the recruitment of immune cells, including neutrophils, macrophages and monocytes to the fracture hematoma. During callus remodelling, mast cells promote osteoclast formation and activity (*Kroner et al., 2017*).

Based on the proposed role of mast cells in osteoporosis as well as in fracture healing, we next investigated whether these cells play a critical role in compromised healing associated with osteoporosis. As expected, in mast cell-competent control mice, OVX enhanced the inflammatory response and reduced osteoblast but increased osteoclast activity in the fracture callus, finally resulting in a poor healing outcome. Notably, the negative effects of OVX on fracture healing were completely abolished in mast cell-deficient mice (*Fischer et al. 2022*). Concluding, mast cells appear to be involved in osteoporosis development and in impaired fracture healing in osteoporotic bone.

Recent studies of our group revealed that mast cells also drive systemic inflammation and compromised bone repair after severe trauma (*Ragipoglu et al. 2022*). This implies that targeting mast cells might be a therapeutic option to improve bone regeneration not only in osteoporotic patients but also in patients with multiple injuries.

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Role of soft tissue in fracture-related infection (FRI) and periprosthetic joint infection (PJI)

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INTRODUCTION: Based upon two decades of clinical orthoplastic experience at the University Hospital Basel, principles and treatment strategies in the field of orthoplastic surgery have been developed. Retrospective orthoplastic data analysis led to the following four cornerstones of treatment: (i) osseous debridement and stabilization, (ii) infection control, (iii) blood flow optimization and (iv) soft tissue reconstruction. These principles can be universally applied and are independent of the cause of the wound. These include traumatic wounds, defects after oncological surgery, diabetic wounds and wounds with exposed hardware in fracture-related infection (FRI) or periprosthetic joint infections (PJI). Continuous long-term orthoplastic outcome is assessed through standardized prospective data collection and patient reported outcome measurement (PROM).
The aim of this invited keynote lecture is to provide an overview of the orthoplastic principles and treatment strategies. In particular it will focus on the role of soft-tissue in FRI and PJI.

METHODS: Patients with a lower leg FRI requiring soft tissue reconstruction (STR) (local, pedicled and free flaps) were included in a retrospective analysis. The main outcome measure was the success rate of STR. Further outcome measures were fracture nonunion and recurrence of infection.
Patients with a PJI of the knee and concomitant extensor apparatus deficiency between 1999 and 2020 were included in another retrospective analysis. This study focussed on the timing and type of soft-tissue reconstruction and the long-term orthoplastic outcome.

RESULTS: One-hundred fourty-five patients with lower leg FRI were identified, of whom 58 (40%) received STR (muscle flaps: n = 38, fascio-cutaneous flaps: n=19; composite osteocutaneous flap: n = 1). In total seven patients required secondary STR due to primary flap failure which was successful in all cases. Out of the 43 patients who completed the 9-month follow-up, 11 patients presented with fracture nonunion and 12 patients with a recurrent infection. Polymicrobial infection was a significant risk factor for fracture nonunion. Primary flap failure was neither a risk factor for compromised fracture consolidation nor for recurrence of infection.
One-hundred sixty patients had PJI after total knee arthroplasty. Plastic surgical reconstruction of a concomitant perigenicular soft-tissue defect was indicated in 47 patients. Of these, six presented with extensor apparatus deficiency. One patient underwent primary arthrodesis and five patients underwent reconstruction of the extensor apparatus. The principle to reconstruct missing tissue 'like with like' was thereby favoured. Despite good functional results 1 year later, long-term follow-up revealed that two patients had to undergo arthrodesis because of recurrent infection.

DISCUSSION & CONCLUSIONS: Patients with FRI and PJI represent a complex subgroup and are rich in complications. They must be treated in a specialized bone and joint infection unit. The earlier the interdisciplinary treatment starts, the better the long-term orthoplastic outcome is. Therebye, the intact soft-tissue envelope is crucial for a successful orthoplastic long-term outcome both in FRI and PJI. The principle of reconstructing tissue 'like with like' provided good functional and aesthetic results, as the replaced tissue contained intrinsic properties that were similar to those of the original tissue.

Thermosensitive support baths promoting aligned anisotropic stem cell clustering for endochondral bone tissue engineering

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INTRODUCTION: Chondrogenic stimulation of spherically clustered stem cells is an established approach in endochondral tissue engineering. However, improved methodologies are needed to spatially organize cell clusters into aligned longitudinal patterns of developmentally relevant length scales. We present a hydrogel support bath composed of poly(N-isopropylacrylamide)-graft-chondroitin sulfate (pNIPAAM-CS) blended with gelatin and Carbopol 940®. We designed the biomaterial to exhibit Bingham plastic fluid-like behaviour at 25°C, enabling bioprinting of cells into embedded anisotropic channels. Additionally, we hypothesized that the cell compatible, temperature-triggered gelation of pNIPAAM at 32°C would provide a thermosensitive surface which could be exploited for stimulating the formation of closely associated and oriented cellular patterns.

METHODS: Human bone marrow derived mesenchymal stem cells (MSCs, P4) were suspended in 6% porcine gelatin bioink at a density of $4x10^6$ cells/mL. The cell suspension was microextruded through a 300 µm (inner diameter) needle into the support bath and cultured in medium supplemented with 10 ng/mL TGF-β1. Constructs were either maintained under static temperature at 37°C or dynamic conditions by cooling to 25°C for 10 min at days 1, 3 and 5. At day 7, constructs were visualized with fluorescent microscopy and a semi-quantitative analysis was carried out with Fiji (NIH, Bethesda, MD, USA) on $n \geq 5$ images per group to characterize the effects of static versus dynamic culture on cell pattern size.

RESULTS: Extrusion of single cell suspension into the support bath created an embedded channel of cells exhibiting a mean lateral pattern width of 390. 42 +/- 113.7 µm (day 0). Dynamic culture induced longitudinal aggregation of the cells along the channels (Fig. 1A) and a decrease in mean width of cell distribution to 180.83 +/- 77.5 µm by day 7 ($p<0.001$, Fig. 1B). On the contrary, width increased slightly to 450.35 +/- 77.5 µm under static conditions ($p>0.05$). Fluorescent intensity distribution profiles showed observable increases in anisotropicity for dynamic versus static culture (Fig. 1C).

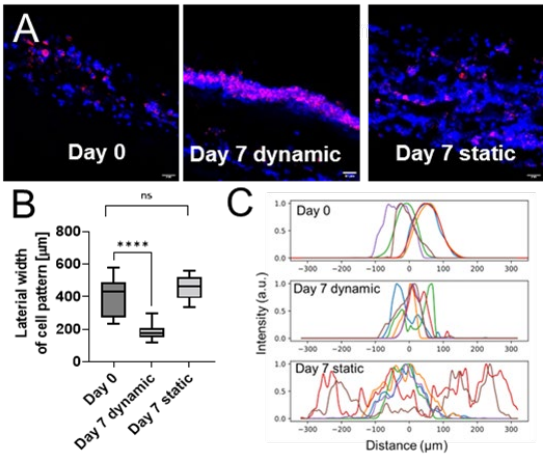

***Fig. 1**: Channel-embedded MSCs cultured under dynamic or static conditions. (A) Cell nuclei stained with DAPI (blue) and TRITC-conjugated phalloidin (pink), (B) Width of cell distribution within the channels, (C) Fluorescent intensity distribution of the cells. Scale bars 50µm.*

DISCUSSION & CONCLUSIONS: Dynamic conditions restricted the lateral width of cellular distribution, possibly due to persistent anchorage deprivation induced by cycling through the critical solution temperature of pNIPAAm. This finding represents an advance over current bioprinting approaches, where the lateral width scale after cell fusion is 300 µm or greater [1,2]. Our novel approach has potential for endowing improved anisotropic organization to engineered bone and other tissues.

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Chronic psychosocial stress disturbs fracture healing via catecholamies produced by immune cells

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INTRODUCTION: Chronic psychosocial stress is associated with negative effects on bone in humans[1]. Using the Chronic Subordinate Colony Housing (CSC) paradigm, a mouse model for chronic psychosocial stress[2], our group previously also demonstrated a disturbed fracture healing in mice exposed to CSC, which was accompanied by an increased expression of tyrosine hydroxylase (TH), the rate limiting enzyme of catecholamine synthesis, in neutrophils in the fracture callus[3]. Therefore, our hypothesis is that the adverse effects of chronic stress on fracture healing are mediated by a locally increased catecholamine production by myeloid cells. The aim of this study was to prove this hypothesis and its translational relevance.

METHODS: 7 – 8-week-old male mice with a specific *TH* knockout in myeloid cells ($TH^{fl/fl}$/CD11b-Cre^{+}) and Cre^{-} control mice were exposed to the CSC paradigm or housed as single housed control (SHC) mice. After SHC/CSC exposure, a femur osteotomy was applied to investigate the different fracture healing phases via FACS, histology and µCT. In a clinical study, the fracture hematoma of patients with upper ankle fracture was collected and analysed for TH expression. Standardized questionaires for psychosocial comorbidities and the patients' mobility 3, 6, 9 and 12 months after fracture were analyzed and correlated with the TH expression via Spearman analysis.

RESULTS: Compared to Cre^{-} SHC mice, Cre^{-} CSC mice showed a disturbed local inflammation mith increased numbers of $CD11b^{+}Ly6G^{+}$ neutrophils in the fracture hematoma 24 h after fracture. Cre^{-} CSC vs. SHC mice further showed decreased numbers of $Runx2^{+}$ chondrocytes in the fracture callus 10 d after fracture, indicating a disturbed chondrocyte-to-osteoblast transdifferentiation, and reduced BV/TV and increased cartilage area in the fracture callus, indicating a disturbed fracture healing. All of these stress-inuced effects on fracture healing were abolished in Cre^{+} mice. The TH expression in human fracture hematoma positively correlated with different mental stress scores as well as with mobility impairment 6, 9 and 12 months after fracture.

DISCUSSION & CONCLUSIONS: Our data indicate a critical role of catecholamines produced by myeloid cells in the mediation of negative stress effects on fracture healing in mice as well as in humans.

ACKNOWLEDGEMENTS: This study was supported by the Collaborative Research Centre CRC1149.

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Analyzing bone regeneration in time and space: Novel approaches for multi-modal, multi-dimensional imaging

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The bone marrow is compartmentalized, containing periosteal, endosteal and vascular niches. On a cellular level, bone regeneration is spatially and temporally orchestrated, and each marrow compartment maintains distinct, but interacting pools of mesenchymal and hematopoietic cell types with unique differentiation and proliferation capacities. We are interested in analyzing the spatiotemporal cross-talk between those compartments, under homeostasis and bone regeneration. To that end, we develop and apply novel imaging tools.

We recently established MarrowShield (MarShie), a tissue clearing and light-sheet microscopy pipeline. By preserving the bone marrow structure, it enables us to analyze the entire marrow, as well as cortical and periosteal regions of long bones in three dimensions, with subcellular resolution. Using MarShie, we detect Prx1+ stromal and CX3CR1+ myeloid cells together with Cdh5+, and CD31+ endothelial cells, not only under homeostatic conditions but also after a drill-hole injury and in an osteotomy model. We observed that CX3CR1+ cells sequester the area of bone injury immediately after tissue damage and are in close contact with blood vessels. At day 14 CD31high vessels are wrapped by Prx1+ mesenchymal cells in the osteotomy gap. This implies that vascular interactions with other cellular compartments in the bone marrow are still underestimated, and that such 3D tools can help to reveal niche specific interactions under different conditions, for example regeneration, aging or cancer.

To enable the analysis of cellular dynamics processes in the bone marrow at a single cell level, we developed a lens implant for the femur, thereby enabling longitudinal intravital imaging in the bone marrow (LIMB). We also combined this implant with an osteotomy, allowing us to monitor single cells over the course of bone regeneration. Complementing our light sheet data, we found that CX3CR1+ myeloid cells enter the fracture gap early after injury, fostering the formation of type H vessels, which are known to promote bone formation. Later, the myeloid cells remain in close contact with endothelial cells, throughout the regenerative process. Based on the hypothesis that the microenvironment influences the metabolic properties of myeloid cells, thereby impacting on their function, we are currently analyzing the metabolic status of both the myeloid cells and their microenvironment in the fracture gap by label-free NAD(P)H fluorescence lifetime imaging (FLIM).

Engineering 3D Human Multicellular Bone *in vitro* Models as Anti-tumor Drug Screening Systems

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INTRODUCTION:

The bone is a complex and dynamic tissue, in which the equilibrium between bone deposition and resorption can be perturbed by various pathological conditions, including bone metastases. Against them, no effective therapy has been developed yet, and available treatments are primarily palliative, aiming at restoring bone homeostasis. To improve the process of anti-metastatic drug discovery, new pre-clinical models are required, since available in vivo and in vitro models are limited by species specific differences in tumor mechanisms and by an oversimplification of the bone environment, respectively. Furthermore, potential side effects can be neglected with available models, resulting into unexpected toxicity of candidate drugs in clinical trials. In this scenario, advanced 3D in vitro models could become relevant assets for research and pharma industry for the discovery of new drugs against bone tumors, overcoming limitations of current models. To this end, our work aims at developing complex 3D in vitro models of bone tissue, taking into account its heterogeneous composition, to be exploited for the test of anti-metastatic drugs.

METHODS:

We firstly developed microfluidic devices including a perfusable microvascular network and reproducing bone and muscle like environments. Then, we improved our model by inserting a vascularized metastastic spheroid connected to the existing vasculature in the bone-like environment. We further generated millimeter-scaled vascularized bone models based on osteoblasts, osteoclasts, vascular cells and mesenchymal stromal cells embedded in a 3D hydrogel loaded with hydroxyapatite nanoparticles. Then we added immune cells and breast cancer metastatic cells, and we tested the effects of anti-tumor drugs in our system, adding rapamycin and doxorubicin, two FDA-approved anti-tumor drugs, known to have side effects on the vascular compartment.

RESULTS:

Using our microfluidic models, we showed how the extravasation of cancer cells was higher in a bone-like environment as compared to a muscle one or an empty matrix. Furthermore, in the presence of immune cells such as neutrophils, the permeability of the vascular network was affected, and immune cells were able to extravasate from the network into the cancer spheroid. In the mm-scaled models, the simultaneous presence of all bone cell types and of the mineral component increased the bone turnover, as compared to simpler culture conditions. Furthermore, tumor cells were able to colonize this bone microenvironment, particularly in the perivascular niche. When antitumor drugs were added to the metastatic bone environment, tumor cell resistance to the drugs was increased by the presence of a bone microenvironment, as compared to a simpler model in which cancer cells were grown as a 3D monoculture. Furthermore, we were able to show the antiangiogenic effects of the drugs, by monitoring the damage to the microvascular network in the model. Finally, we tested our 3D bone model also with cells deriving from Ewing sarcoma, a pediatric bone tumor, showing that they could proliferate in our mineralized bone model. Sarcoma cell viability was affected by the knockdown of relevant genes and by the addition of oxidative stress inducers, in accordance with results shown in mouse experiments.

CONCLUSION:

Overall, the screening of anti-metastatic drugs in an in vitro model recapitulating the complexity of bone environment allowed to better estimate the effects of potential drugs both on their intended target and on other components of the microenvironment as compared to simpler models.

Alterations of bone microarchitecture and bone fragility in a type 1 diabetes mouse model (Akita mice)

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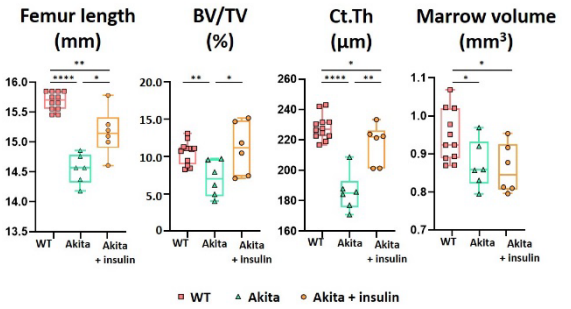
INTRODUCTION: Type 1 diabetes (T1D) is recognized as a major risk factor for fragility fractures [1], with up to a 6-fold increase in risk [2] and a delay in fracture healing [3]. Increased fracture risk is due to a low bone mineral density [4] and to an impaired microarchitecture and bone strength [5]. Despite the initiation of insulin therapy upon diagnosis, diabetic bone fragility appears to persist into adulthood. Hence, we aim to characterize the diabetic bone phenotype of a spontaneous mouse model of T1D (Akita mice) and to evaluate the effects of insulinotherapy at T1D onset on bone microarchitecture and strength.

METHODS: Due to spontaneous mutation in the insulin 2 gene, heterozygous male C57BL/6-Ins2^{Akita}/J (Akita$^{+/-}$) mice develop T1D by the age of 4-5 weeks. The Akita diabetic phenotype includes severe hyperglycaemia, hypoinsulinemia, polydipsia and polyuria without obesity. Upon onset of diabetes, Akita mice were treated (or not) with a sustained-release insulin implant (2 implants/mouse, subcutaneously, release of 0.1 U/day/implant for 30 days, LinShin, Canada) for 8 weeks. Bone microarchitecture was assessed *ex-vivo* by µ-computed tomography (Scanco Medical, Switzerland) on femur, tibia and L2 vertebrae.

RESULTS: 14 weeks-old Akita$^{+/-}$ mice have a lower body weight (-25.4%), higher water consumption than WT littermate mice and important polyuria and urinary glucose excretion. Interestingly, Akita$^{+/-}$ mice display a shorter femur (-7.1%) and tibia (-3.4%) than normoglycaemic WT mice. Besides, in both femur, tibia and spine, Akita$^{+/-}$ mice have a lower bone volume fraction (BV/TV) (-33.2% for tibia), cortical thickness (Ct.Th) (-18.6% for tibia) and endosteal perimeter (-7.9% for tibia) than WT mice. Akita$^{+/-}$ mice under insulinotherapy rapidly show an improvement in fasting glycaemia and T1D symptoms (polydipsia, polyuria). Insulin treatment allows a total recovery of the body weight and a partial rescue of bone length (+4.2% for femur). Regarding microarchitecture, insulin treated Akita$^{+/-}$ mice displayed a similar BV/TV and Ct.Th than WT mice, in both femur and tibia, while no change in endosteal perimeter was observed compared to Akita$^{+/-}$ mice.

*Fig. 1: Effect of type 1 diabetes and insulin treatment on bone microarchitecture (femur length and tibia BV/TV, Ct.Th and endosteal perimeter). *p<0.05, **p<0.01, ****p<0.0001*



DISCUSSION & CONCLUSIONS: Akita$^{+/-}$ mouse model is a mildly severe model of T1D, with an early onset of the disease. The Akita bone phenotype is characterized by an altered trabecular and cortical bone microarchitecture, with defects in bone growth. Insulinotherapy restores partially the growth defects and significantly improves the trabecular and cortical bone parameters, by promoting periosteal bone formation. Decrease in cortical thickness and trabecular bone volume in T1D mice could contribute to the increased bone fragility observed in Akita mice [6]. Thus, insulin deficiency and/or bad glycaemic control appear to be a major cause of diabetic bone fragility in the Akita mouse model.

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Biomimetic alginate-based scaffolds with bioactive glass particles as a relevant *in vitro* model of bone tissue

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INTRODUCTION: Three-dimensional (3D) *in vitro* models for cell culture have great potentials to overcome drawbacks of traditional two-dimensional systems by providing more realistic conditions in the cell microenvironment *in vitro* and therefore more reliable results. One of the approaches is based on using scaffolds for providing 3D structures for cell attachment. In this work, scaffolds based on alginate with bioactive glass particles were developed and tested as a 3D cell culture platform imitating bone tissue composition and structure. Murine osteosarcoma cells were used as a model of bone-related cells.

METHODS: Two types of melt-derived bioactive glasses (BAG), SBA2 ($48SiO_2$-$18Na_2O$-$30CaO$-$3P_2O_5$-$0.43B_2O_3$-$0.57Al_2O_3$ mol% [1]) and 47.5B ($47.5SiO_2$-$10Na_2O$-$10K_2O$-$10MgO$-$20CaO$-$2.5P_2O_5$ mol.% [2]), were examined regarding biocompatibility in the direct contact test with K7M2-wt osteosarcoma cell line as well as bioactivity in terms of hydroxyapatite (HAP) formation in culture medium over time analyzed by FE-SEM and EDS. Macroporous composite scaffolds were produced by controlled gelation of 2 wt.% Na-alginate solutions containing 2 wt.% BAG (SBA2 or 47.5B) and $CaCl_2$ in the concentration range 0.045-0.075 wt.% poured in dialysis tubes and placed in 3 wt.% $CaCl_2$ solution, followed by freeze-drying and rehydration. The obtained cylinders were cut into discs (10 mm diameter, 4 mm thick) and characterized in terms of porosity. Scaffolds with optimized porosity and pore size distribution were then tested for cultivation of K7M2-wt osteosarcoma cells. After optimization of the cell seeding procedure, the seeded scaffolds were cultivated for 7 days under static conditions. The MTT test was performed before and after the experiment and scaffolds were cross-sectioned and H&E stained for histological analyses.

RESULTS: Both bioactive glasses were shown to be biocompatible by the MTT test after 1 and 3 days in the direct contact test. FE-SEM and EDS analyses revealed that 47.5B BAG particles were fully covered with newly formed HAP crystals after 5 days in the culture medium, while SBA2 particles were partially covered with amorphous CaP phase after 8 days. Macroporous composite alginate scaffolds were successfully produced with both BAG types. Pores had lamellar shape and optimal porosity and size distribution were achieved with the Ca^{2+} content of 0.06 wt.% in the initial solution. Scaffolds made with 47.5B particles were selected for further experiments due to higher bioactivity. By optimization of the cell seeding procedure, seeding efficiency of >90% was achieved while the static culture has shown higher cell metabolic activity after 7 days of cultivation as compared to the initial samples.

DISCUSSION & CONCLUSIONS: First results obtained in this work show great potential of the developed composite scaffolds for cultivation of osteosarcoma cells as a model of bone-related cells. Further experiments are intended to investigate bioactivity of BAG within the scaffold so to further characterize the *in vitro* cell microenvironment as well as to assess effects of active HAP formation on cultured cells in comparison to cell cultivation in scaffolds without BAG.

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A 3D cell culture model mimicking pathological and physiological conditions in bone

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INTRODUCTION: Three-dimensional (3D) cell culture models aim to recapitulate native cellular environment offering an innovative approach to addressing various obstacles inherent to traditionally utilized cell monolayers and animal models. Therefore, the application of 3D models is highly encouraged in different fields such as tumor research, antitumor drug testing, personalized medicine and tissue engineering. Our 3D *in vitro* bone model consists of a bone-like scaffold and perfusion bioreactor, which provide cells with enhanced mass transfer and adequate biomechanical stimuli *i.e.* hydrodynamic shear stresses. The model is aimed to imitate both bone pathological conditions (osteosarcoma) and bone physiological conditions when representative cell types (mesenchymal stem cells, osteoblasts) are employed. Here, we provide an overview of the engineering aspects of our model and the roles these aspects may have in shaping the cell culture.

METHODS: Bone-like scaffolds were obtained by ionic gelation of the mixture of alginate (2 wt.%) and hydroxyapatite particles (2 wt.%) followed by freezing and lyophilization. Scaffold porosity and pore distribution were determined by using ImageJ software for analysis of the scaffold cryo-sections. Osteosarcoma model was established by seeding the scaffolds with murine osteosarcoma K7M2-wt cell line, whereas to model bone physiological conditions human bone marrow-derived mesenchymal stem cells were used. In both models, cells were cultivated for 7 days in a perfusion bioreactor (3D Perfuse, Innovation Center of the Faculty of Technology and Metallurgy, Belgrade, Serbia) at the medium flow rate of 0.27 cm^3/min, corresponding to the superficial velocity of 40 μm/s. Cell cultures under static conditions served as controls. Cell metabolic activity was estimated by MTT/resazurin assay and morphology was examined by histological analyses. Shear stresses acting on the cells were calculated based on analyses of histological sections by using a cylindrical pore model.

RESULTS: The obtained scaffolds were 60% porous with heterogeneous pore sizes, macropores being dominantly present. Both cell types adhered to the scaffold pore surface (cell seeding efficiency >80%) and exhibited high metabolic activity when cultured in perfusion bioreactors. During the short-term cultivation, osteosarcoma cells formed spheroids, which were more compact in perfusion cultures than in static, whereas mesenchymal stem cells aligned along the pores. Estimated shear stresses acting on the cells in perfusion cultures were in the order of 5 - 20 mPa.

DISCUSSION & CONCLUSIONS: Scaffold porosity and pore interconnectivity contributed to the uniform cell distribution across the scaffold volume. Cell cultures in perfusion bioreactors exhibited higher metabolic activity and a more pronounced ability for self-assembly, which is attributed to enhanced mass transfer and the physiological range of shear stresses. In future studies, the effects of shear stress magnitudes on cell self-assembly in perfusion cultures together with expression of the specific genes will be investigated. The 3D model system exhibited high potentials for supporting the cultures of both osteosarcoma and osteogenic cells.

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β-TCP from 3D-printed scaffold can act as an effective phosphate source during the osteogenic differentiation of human mesenchymal stromal cell

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INTRODUCTION: Human bone marrow derived mesenchymal stromal cells (hBM-MSCs) are often combined with calcium-phosphate-based 3D-printed scaffolds with the goal to repair bone defects. *In vitro*, the induction of osteogenic differentiation requires, among other supplements, the addition of organic β-glycerophosphate (BGP), which acts as a phosphate source. However, the ability of MSCs to use phosphate contained within the 3D-printed scaffolds during *in vitro* osteogenesis is poorly understood. The aim of this study is to investigate whether the phosphate contained within 3D-printed scaffolds is sufficient to act as phosphate source during the osteogenesis of hBM-MSCs.

METHODS: hBM-MSCs (obtained will full ethical approval) of five independent donors are seeded on top of 3D-printed discs composed of poly(lactic-co-glycolic acid) (PLGA) and β-tricalcium phosphate (β-TCP) and kept for 28 days under three different culture conditions: 1) basal medium, 2) osteogenic (basal medium supplemented with dexamethasone, ascorbic acid and BGP) and 3) osteogenic medium without BGP. Alkaline phosphatase (ALP) activity - and staining are performed after 14 days of culture. Mineral deposition is stained after 28 days of culture using OsteoImage™ Mineralization Assay.

RESULTS: Mean ALP activity is upregulated in the osteogenic group compared to the osteocontrol group, and the subtraction of BGP from the osteogenic medium shows an even higher upregulation (Figure 1A). ALP staining at day 14 (Figure 1B) and fluorescent-stained mineral deposition at day 28 (Figure 1C) show visibly increased ALP and mineral deposition, respectively in both the osteogenic groups compared to the osteocontrol group. No significant differences are observed between the two osteogenic groups for the presented donors. The subtraction of BGP maintains or upregulated the expression of osteo-relevant markers for hBM-MSC *in vitro* osteogenesis.

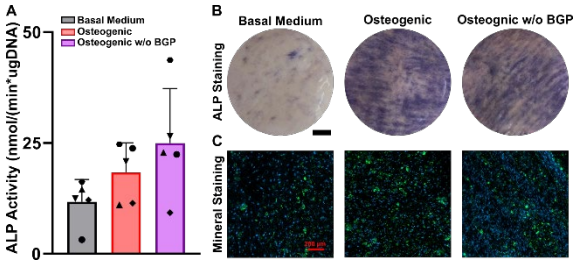

Figure 1. Osteogenic assessment of hBM-MSCs of five independent donors cultured on 3D-printed discs under three conditions: basal medium, osteogenic medium and osteogenic medium without β-glycerophosphate (BGP) for 28 days: A) Alkaline Phosphatase (ALP) activity normalized to DNA content at day 14, each data point represents each individual donor: donor A (●), donor B (▲), donor C (♦), donor D (⬢) and donor E (▼), B) Representative image (donor A) of ALP staining at day 14, scale bar 2,5 mm and C) Representative image (donor C) of mineral deposition (green) and nucleus (blue) at day 28.

CONCLUSIONS: Results suggest that hBM-MSCs can hydrolyze ionic bonded phosphate from the β-TCP and use it during extracellular matrix mineralization, which makes the addition of BGP to the culture medium redundant. β-TCP embedded within personalised 3D-printed scaffolds can simplify *in vivo* osteogenesis to improve the clinical intervention of repairing bone defects

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RIA VERSUS BMC AS ORTHOBIOLOGIC AUGMENTS TO ALLOGRAFTS

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Stoker AM, Stannard JP, Cook JL. Reamer-irrigator-aspirate versus bone marrow aspirate concentrate for osteoprogenitor cell retention and osteoinductive protein release on cancellous bone. J Orthop. 2021 Aug 9;27:13-16. doi: 10.1016/j.jor.2021.08.004. PMID: 34434001; PMCID: PMC8371145

Introduction: This study was undertaken to determine if bone harvested with RIA (Reamer-Irrigator-Aspirator) is associated with significantly higher osteoprogenitor cell concentration and osteoinductive protein elution compared to bone marrow aspirate concentrate (BMC) when cultured on human cancellous allograft.

Methods: With Animal Care and Use Committee approval (ACUC #9167), both BMC percutaneously harvested from iliac crest (7%) and 10-mm RIA from ipsilateral femur (3 passes) were collected from skeletally mature purpose-bred hounds (n = 3, F). ~250 µL of BMC or RIA were used to saturate 2 cancellous allograft bone cubes per dog. Bone cubes (n = 6/type) were cultured individually in a 6-well plate for 7 or 14 days. On days 7 and 14 (n = 3/type/time point) cellular adherence to the bone block was determined using the microscopic cell viability stain calcein AM, and cellular adherence to the plate well was assessed. On day 3, 7, and 14 media were collected and assessed for leptin, platelet-derived growth factor (PDGF)-AA, PDGF-BB, SOST, vascular endothelial growth factor (VEGF), DKK-1, fibroblast growth factor (FGF)-23, osteocalcin, osteoprotegerin (OPG), osteopontin (OPN), and adrenocorticotropic hormone (ACTH).

Results: Media Biomarker Concentration On day 3, BMC had significantly higher concentrations of DKK-1, EGF, OPN, and OPG compared to RIA. On day 7 BMC only had significantly higher concentrations of DKK-1, however RIA had a higher concentration of PTH. On Day 14 there were no significant differences in the concentrations between RIA and BMC. Bone Block Cell Adherence On day 7 and 14, BMC had extensive cell adhesion to bone block surface and infiltration into block. On the other hand, RIA had minimal cell adhesion to bone block surface and no infiltration into block. Well Surface Cell Adherence On day 14, BMC had both cell adhesion and expansion compared to RIA with no cell adhesion or expansion.

Conclusion: In this canine in vitro model with human allograft bone cubes, BMC had significantly higher concentrations of DKK-I, EGF, OPN, and OPG, while RIA had a significantly higher concentration parathyroid hormone. Furthermore, BMC had a higher number of progenitor cells on the bottom of the well and on the allograft cubes. These results were somewhat unexpected and prove the null hypothesis. To see if this holds true in humans, we are in the process of performing a prospective human clinical study. These results will help