POLITECNICO DI TORINO

Master's Degree in Biomedical Engineering
Bionanotechnologies
A.a. 2023/2024
Degree session December 2024

Master's Degree Thesis

ESTABLISHING AN ORGANOTYPIC BONE FRACTURE MODEL FOR OSTEOGENESIS IMPERFECTA

Supervisors:
Prof. Raffaella Sesana
Prof. Dr. Ralph Müller
Prof. Laura Maria Vergani

Advisors:
Dr. Gian Nutal Schädli
Dr. Federica Buccino

Candidate:
Marco De Asmundis 308946

Alle mie nonne,

ACKNOWLEDGEMENTS

I would like to thank Prof. Raffaella Sesana, Prof. Laura Maria Vergani and Dr. Federica Buccino for giving me the opportunity to carry out this thesis at ETH Zürich. Here, I was welcomed by Prof. Dr. Ralph Müller, Gian Nutal Schädli and the entire Lab for Bone Biomechanics. They shared with me their knowledge and their research, and I am honored to have been part of their extraordinary group.
Thank you, Mum and Dad, since you trusted me every day, from the very beginning of this journey, I am not writing about university but about life. Thank you also to my aunt Elisabetta with Marco, my uncles Diego and Ricci, and my cousins Federica with Erik and Lorena. I always enjoyed the time spent with you. Thank you to the members of the ten-year-old Triade, Luca and Matteo, I hope our friendship will be enriched by many other good moments together. Thank you of course also to the girls of "Mare?", Carlotta, Eleonora, Chiara and Eleonora (Mini-Ele), you always made my holidays, trips, birthdays and nights valuable. I want to acknowledge my friends from the first year of Poli: Davide, Eric, Luca and Nicole. In these years, we have been keeping in touch, and this fact witnesses our bond.
Thank you, Francesco (Gigi), since we demonstrated that our friendship is stronger than distance and thank you Niccolò for the countless nights together here and there. I want to acknowledge some Italian friends, Antonello, Matteo and Paolo, which made some Swiss weekend special. Finally, thank you to Mauro, the first friend who visited me during my thesis, and to the rest of BASTA team: Andrea, Giulia, Linda, Silvia and Stefano.

Marco

Table of contents

1 Abstract

Osteogenesis imperfecta (OI), also known as brittle bone disease, is a genetically and phenotypically heterogeneous collection of rare genetic disorders. It is characterized by increased bone fragility, which results in frequent fractures, especially during childhood. Most cases are caused by mutations in either COL1A1 or COL1A2, the genes encoding the alpha-chains of type I collagen, the main organic part of bone. However, in recent years, new mutations in more than twenty collagen-related genes have been found to cause OI. Despite the different causes, hypermineralization is a common feature of the great majority of OI cases. Current treatments, such as bisphosphonates, often fail in preventing bone fractures. This fact could be attributable to shortcomings of OI models. Indeed, animal models fall short in recapitulating the genetic and molecular characteristics of the human condition. *In vitro* organotypic bone models represent a promising tool for targeted and personalized medicine, as they can be developed employing patient-derived cells. In this study, we used mechanically loaded 3D-bioprinted patient-specific organoids and weekly micro-computed tomography to investigate the effects of teriparatide, a drug which is gaining attentions in OI management. Moreover, we also used the same tools to assess the altered mineralization in COL1A2-related OI compared to metabolically healthy controls. The results demonstrated an improvement in the dynamic stiffness of OI treated organoids, but there was no difference for the mineralization parameters. Moreover, the results showed only limited evidence of the hypermineralization feature in OI samples when compared to metabolically healthy controls. Overall, patient-derived organotypic bone models proved being a promising tool for disease modelling and testing therapeutic interventions.

2 Introduction

Osteogenesis imperfecta (OI), also known as brittle bone disease, is a genetically and phenotypically heterogeneous collection of rare genetic disorders. Individuals affected by OI have low bone mass, accompanied by bone fragility which results in frequent fractures, especially during childhood. In addition, OI patients often present bone deformities, growth deficiencies and other symptoms such as dentinogenesis imperfecta and blue sclerae. Considering the different clinical manifestations found in OI individuals, they have been categorized into four groups. According to this classification, type I is the mildest condition, type II is the most severe and often results in perinatal lethality, type III is a severe form while type IV is a moderate form. Most (85%-90%) of OI cases are caused by dominant autosomal mutations in either COL1A1 or COL1A2, the genes which encode for the alpha-chains (α1 and α2) of type I collagen, the main organic component of bone matrix. These mutations may lead to either quantitative (due to haploinsufficiency) or qualitative (aberrant structure) defects of collagen molecule, which impact on bone characteristics [1]. Over the years, mutations in more than 24 different causative genes have been identified. These are genes which encode proteins involved in pathways which are crucial for bone development. Although the huge heterogeneity in the causes and disease trajectories of OI, most of the affected individuals share similar modifications in the feature of bone tissue. Indeed, the level of mineralization was found to be increased in almost every type [2]. Notably, the hypermineralization of the bone material is considered as the main contributor to the increased brittleness [3].

Now, OI is a disease without a cure, which means that no current solution can correct genetic defects which cause the disorder. Indeed, pharmacological treatments only aim at managing the symptoms, above all reducing fractures. However, bisphosphonates, the most commonly used compounds, showed only limited evidence in their ability in preventing fractures in adults, while none in children [4]. This situation is attributable to the drug development pipeline, which relies on animal (mainly murine) models, which, despite providing valuable insights, often fall short in recapitulating the genetic and molecular characteristics of the human condition. Moreover, animal models have demonstrated several disadvantages, such as their costly and time-consuming nature, in addition to ethical issues [5].

Organotypic bone models (organoids) hold promise in studying bone disease such as OI, since they are in vitro models that most closely resemble the in vivo situation, providing a cutting-edge approach towards personalized medicine [6]. Indeed, they can be developed using patient-

derived cells, for example exploiting 3D bioprinting, a technique which allows precise placement of cells and biomaterials (ink) in a layer-by-layer process [7]. These organoids have the aim of modelling the process of early bone formation (woven bone), recapitulating the condition of bone healing following a fracture. The Lab for Bone Biomechanics (LBB) have made important steps in the development of bioprinted organoids, optimizing, in a series of publications, a bioink composed by human mesenchymal stromal cells (hMSCs), gelatine, alginate and graphene oxide (GO) [8–10]. However, the employment of hMSCs is not ideal, since the harvesting of this cell population would require a dedicated surgery. Thus, LBB established a pipeline for developing 3D bioprinted human bone-derived cell-laden organoids [11]. In their next work, they applied this pipeline to develop an organotypic bone model for FKBP10-related OI, employing patient-derived cells [12]. Their notable results showed that OI organoids have higher bone volume and higher tissue mineral density compared to healthy controls.

As said, such models could be used to test therapeutic interventions in a personalized manner. One drug which is gaining attentions in the OI management is Teriparatide. It is a recombinant form of the first 34 amino acids (constituting the active part, Figure 2.1) of Parathyroid hormone, a substance which produced *in vivo* by the thyroid glands. In light of its anabolic properties, it is currently used as a drug for osteoporosis [13].

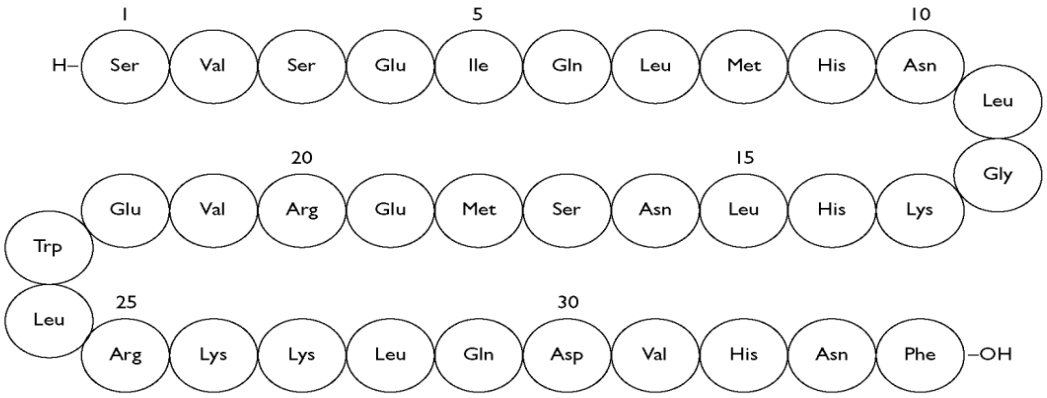

Figure 2.1 Amino acid sequence of teriparatide [13]

In osteoporotic patients, it has been found to have better results, when compared to bisphosphonates, in terms of prevention of fractures [14]. Moreover, Teriparatide has gained attention since it acts directly on osteoblasts, promoting bone deposition, while bisphosphonates act on osteoclast, avoiding bone resorption. Indeed, this latter strategy can be dangerous since

it could mean avoiding the resorption of micro-cracked bone. Moreover, bisphosphonates have raised some concerns due to possible long-term incorporation in bone, that there would not be using teriparatide [15].

Significantly, the use of Teriparatide has been tested is three clinical trials in Osteogenesis Imperfecta patients. The first study included 79 adult patients with OI types I, III and IV and it demonstrated positive effects in the Bone Mineral Density (BMD) at the spine and the hip compared to placebo. Moreover, the effects on bone remodeling markers were similar to studies where teriparatide was tested in osteoporotic patients. However, deeper analysis considering the different OI types revealed that the benefits of the treatment were pronounced in type I OI patients but almost absent in types III and IV OI patients. The authors claimed that it is possible a differential treatment response in patients with quantitative defect of collagen (as in type I) compared to patients with qualitative defect (as in types III and IV). The results of other two studies [16, 17] showed a promising role of teriparatide in the treatment of type I OI adult patients [18]. Furthermore, a fourth clinical trial using teriparatide, followed by zoledronic acid, is still ongoing and it is expected to report in April 2025 [19].

Moreover, Teriparatide has gained attentions as a promoter of fracture healing [20], and for this reason it was also employed in three case studies for the treatment of atypical femoral fracture in OI individuals. The authors claimed that teriparatide could effectively promote bone healing in different OI types, from mild to moderate forms (type I and IV) [21–23].

In the present thesis, the goal is to develop a patient-specific organotypic bone model for OI. Employing human primary osteoblastic cells derived from a paediatric type III/IV COL1A2-related OI patient and a metabolically healthy control, 3D extrusion bioprinting is exploited to fabricate the bone organoids. The organoids are cultured for a six-weeks period with mechanical loading applied in dynamic compression bioreactors. Fifteen days after the printing, the drug (teriparatide) testing starts and the samples from both groups (OI and healthy) are further subgrouped (treated and control). Teriparatide is administered three times per week.

The effects of drug treatment on bone organoid fidelity, overall mineral formation, organoid mineral density and dynamic stiffness are investigated.

The hypothesis at the base of the study is that drug treatment is expected to favour the mineralization of bone, increasing bone organoid fidelity. The drug should also increase the mechanical properties of the organoid. Furthermore, OI untreated samples are hypothesized to

have higher values in mineralization parameters when compared to Healthy untreated samples, fact which would reflect the *in vivo* feature.

3 Materials and Methods

3.1 Cell expansion

One cryovial containing five million hOB (Passage 4) was taken from liquid nitrogen where it was stored and rapidly thawed in a 37 °C waterbath. Then, its content was added to high glucose control medium (high glucose DMEM, 10% FBS, 1% Anti-Anti), which was inserted into triple flasks and kept for one week in an incubator at 37 °C, 5% CO_2. After the expansion week, cells were detached using 0.25% trypsin-EDTA and frozen at -80 °C, in 12 mL of freezing medium (10% DMSO in FBS) after dividing in 10 cryovials.

Three days after, the previous procedure was repeated but in place of the high glucose control medium, high glucose expansion medium (high glucose DMEM, 10% FBS, 1% Anti-Anti, 1% NEAA, 1 ng/mL bFGF) was used. On the printing day, cells were detached from the triple-flasks using 0.25% trypsin-EDTA, counted with the haematocytometer and then centrifuged for 10 min, at 300g and 4 °C. They were finally resuspended in low glucose control medium (low glucose DMEM, 10% FBS, 1% Anti-Anti), at a concentration of five million cells in 60 µL.

3.2 Hydrogel preparation

For what concerns the hydrogel preparation, this was performed following a previous literature work [10]. The components used were: alginic acid solution salt (Mw = 10,000-600,000) which was purchased from AppliChem GmbH (Darmstadt, Germany), type A gelatin from porcine skin (Mw = 50,000-100,000) which was purchased from Sigma Aldrich (Buchs, Switzerland), glycerol (99.5%) which was purchased from Hänsler AG (Herisau, Switzerland), phosphate buffered saline (PBS) tablets which were purchased from Medicago (Uppsala, Sweden) and 5 mg/mL graphene oxide (GO) dispersion in water solution which was purchased from RoyalElite RoyCarbon (Shanghai, China).

To obtain 8 mL of GO/alginate/gelatine (0.1%/0.8%/4.1% w/v) hydrogel, firstly, 1.6 mL GO solution was ultrasonicated and then mixed with 5.6 mL 1.29x PBS solution (previously prepared dissolving 1 PBS tablet in 777 mL ultrapure water and then autoclaving it) and 0.8

mL glycerol to prepare a homogeneous solution. After that, 70.4 mg alginate and 360 mg gelatine were dissolved in the above GO/PBS/glycerol solution at 50 °C for one hour in a water bath while being agitated by a magnetic fish on a magnetic stirrer at 300 rpm. Then, the mixture was kept overnight in another waterbath, without stirring, to homogenize and fully dissolve the polymers. Then, it was pasteurized at 70 °C for 1 h and finally stored in the fridge at 4 °C.

3.3 Bioprinting

On the printing day, the hydrogel was magnetically stirred for 5 min in a 37 °C waterbath at 400 rpm, and 2 mL were pipetted into a small glass bottle, where also 200 µL of cell suspension and a magnetic fish were added. The small glass bottle was then closed with its lid and put on a magnetic stirrer for 1 min at 200 rpm at room temperature. In the meantime, the bottom side of a polyethylene injection cartridge (3 cc, printing syringe) was closed with a blue cap. When the minute passed, the small glass bottle was opened and its content was directly poured into the printing syringe, making sure to remove the magnetic fish with tweezers as it could prevent the transfer of the bioink from the glass bottle to the printing syringe. The bioink was let flow down on the blue cap and a white cap was added into the syringe and pushed with a spatula until the bioink reached the bottom of the syringe. The top side of the syringe was then closed with another blue cap, and the syringe was transferred in an ice bath for three minutes. After this step, the syringe was transferred to the printing device, a 3Ddiscovery bioprinter (RegenHu; Villaz-St-Pierre, Switzerland). Then, the two blue caps were removed, and a 27-gauge tapered tip (Nordson EFD, Vilters, Switzerland) was installed. The syringe was finally loaded on the bioprinter device. Notably, during the printing of healthy samples, the average room

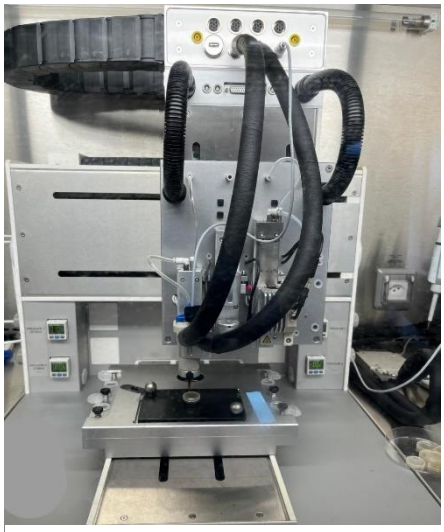

Figure 3.1 3D Discovery bioprinter during a sample printing.

temperature was 21.8 °C and samples were printed with an average pressure of 105 kPa. OI samples were printed with an average room temperature of 23.7 °C, employing an average pressure of 97 kPa.

A lattice-rod structure (10mm x 10mm x 2.5 mm) was used to print the samples onto sample holders, previously coated with an alginate coating. Each sample took around eight and a half minutes to be printed.

After each sample printing, the sample holder, with the sample attached, was taken with tweezers, and put in a crosslinking bath at room temperature in a six-well-plate, ensuring that the entire sample was submerged.

Regarding the crosslinker solution, it was prepared into two steps. First, 5 g of granular $CaCl_2$, purchased from VWR International (Dietikon, Switzerland), were dissolved into 100 mL of ultrapure water to obtain a 5% w/v $CaCl_2$ solution. Then, the $CaCl_2$ solution was added to 150 mL of low glucose control medium (low glucose DMEM, 10% FBS, 1% Anti-Anti) to obtain a 2% w/v $CaCl_2$ solution for crosslinking. Then, the six-well-plates were prepared as it is possible to see in Figure 3.2, two wells contained 8 mL of the 2% crosslinking solution (CL), while the remaining four wells contained 8 mL of low glucose control medium (CM).

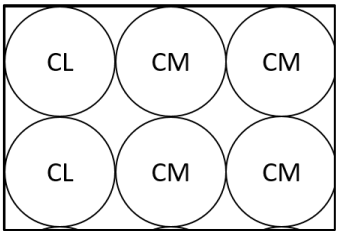

Figure 3.2 Six well plate set-up for crosslinking

After 10 min in the crosslinking solution, the samples were moved with tweezers to the next two wells to be washed twice (for ten seconds each) in low glucose control medium to remove excess of the crosslinker solution. Finally, the samples were moved with tweezers to another six-well-plate containing low glucose control medium and incubated at 37 °C, 5 % CO_2 for one day.

3.4 Dynamic culture

The day after printing, the samples were moved in custom-made polyetherimide bioreactors (one sample per bioreactor) and here cultured with 5 mL of standard osteogenic medium (low glucose control medium, ascorbic acid 50 µg/mL, dexamethasone 100 nM, β-glycerophosphate 10 mM). From this moment forward, medium was changed three times per week (Monday-Wednesday-Friday) opening a hole on the lid, which was normally closed with a stopper.
After each medium exchange, the samples were subjected to cyclic mechanical loading three times per week (Monday-Wednesday-Friday) thanks to two self-made Mechanical Stimulation Units (MSU), Figure 3.3, controlled by LabVIEW (National Instruments, Austin, Texas).

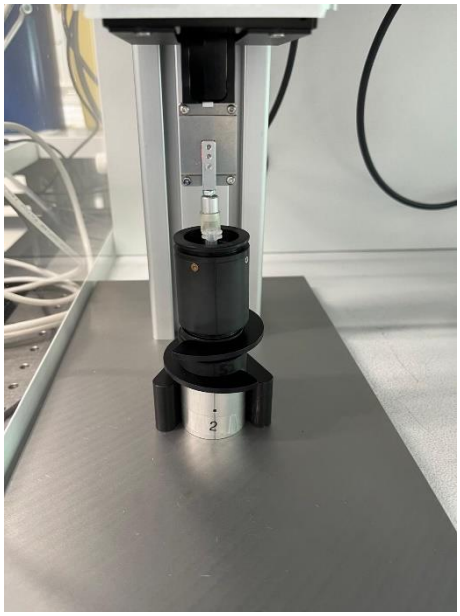

Figure 3.3 Mechanical stimulation unit with inserted bioreactors

This MSU allowed controlling the force $F_{contact}$ to perform the height measurement as well as frequency and strain of the compressive loading regime. In this project a $F_{contact}$ of 0.05 N was applied to measure the sample height. After setting a preload of 20% (15% from the second week forward) strain (which was possible thanks to the just measured height), the loading protocol was applied. This consisted in loading at 1% strain, at 5 Hz for 5 minutes. After the mechanical loading, bioreactors were always immediately placed back in the incubators.

3.4.1 Drug treatment

From each group (OI and healthy), samples were subgrouped for the drug study, based on the printing quality. Two days before the drug study started, standard osteogenic medium was exchanged with osteogenic medium without dexamethasone (low glucose control medium, ascorbic acid 50 µg/mL, β-glycerophosphate 10 mM). This medium was then used until the end of the drug study. The drug study started on day 15 after printing the bone organoids and the drug was administered three times a week (Monday-Wednesday-Friday).

On the drug testing days, one 10 µM stock aliquot was thawed and 20 µL of it were mixed with 980 µL ultrapure water to get 1 mL of a 0.2 µM working solution. The working solution was then syringe filtered using a 1-mL syringe and a 0.22 µm filter. After that, 5 µL of the working solution were added into the 5 mL culture medium of the treatment group. This resulted in a concentration of the drug on cells equal to 0.2 nM. On the other side, the samples of the control group received 5 µL of syringe-filtered ultrapure water. After the treatment, the medium was mixed five times using a 1-mL pipette. Two hours after the treatment, medium was changed in both groups using osteogenic medium without dexamethasone.

3.5 Micro-computed tomography

The mineral formation was investigated by *in situ* time-lapsed micro-computed tomography (micro-CT or µCT) scanning with a µCT 45 micro-CT scanner (SCANCO Medical AG, Brüttisellen, Switzerland).

Fig. 2.2 Micro-CT scanner.

The bioreactors were weekly scanned at a voxel resolution of 34.5 μm, with an energy of 45 kVp, intensity of 177 μA, and an integration time of 600 ms. Each scan lasted approximately 36 min. Scan visualization and analysis were done using the manufacturer's software (SCANCO Medical, Brütisellen, Switzerland). The reference line for the micro-CT scan was 51702 μm.

3.6 Fixation and embedding

At the end of the dynamic culture using osteogenic media, all the bioreactors were disassembled and the samples on their stages were placed in six-well-plates. Here, they were washed twice with PBS. Then, organoids were fixed in a 4% PFA in 10 mM $CaCl_2$ and 150 mM NaCl solution for about two hours at room temperature under the fume hood. Next, they were rinsed twice (1 min for each rinsing) in a 10 mM $CaCl_2$ and 150 mM NaCl solution. The, they were cryoprotected in a 10% sucrose with 10 mM $CaCl_2$ solution for two hours. After this step, they were further cryoprotected in a 30% sucrose with 10 mM $CaCl_2$ and 150 mM NaCl solution for at least 16 hours. Finally, the samples were cryoembedded in OCT compound which was purchased from VWR International (Dietikon, Switzerland). To perform this last step, each sample was carefully removed from its sample holder, dried shortly with a tissue and placed, upside down, in a Tissue-Tek well containing OCT compound. If necessary, OCT compound was added to cover the sample. The wells were flash-frozen by placing on a metallic surface immersed in a liquid nitrogen bath in a Styrofoam box. The wells were then inserted in a plastic bag which was stored in a fridge at -80 °C until the cryosectioning.

3.7 Statistical analysis

Statistical analysis was performed using MATLAB. *p*-values less than 0.05 were considered statistically significant. Welch's t-test was conducted to assess the impact of drug treatment and to evaluate differences between OI and healthy untreated groups.

4 Results

4.1 Mineralization of patient-derived organoids

To investigate the mineralization of patient-derived organoids, weekly µCT scans were performed. Figure 4.1 shows the 3D renderings of the week-6 scans of the entire sample cohort. Considering technical issues, such as bad printing quality or suboptimal handling, some samples were excluded for the following analysis. In particular, sample 2 was impossible to be properly loaded from week 2, sample 54 was bad printed. Similarly, samples 3, 58 and 62 were not fully good printed, while for samples 10 and 11, the piston fell during the loading positioning, causing a sharp break.

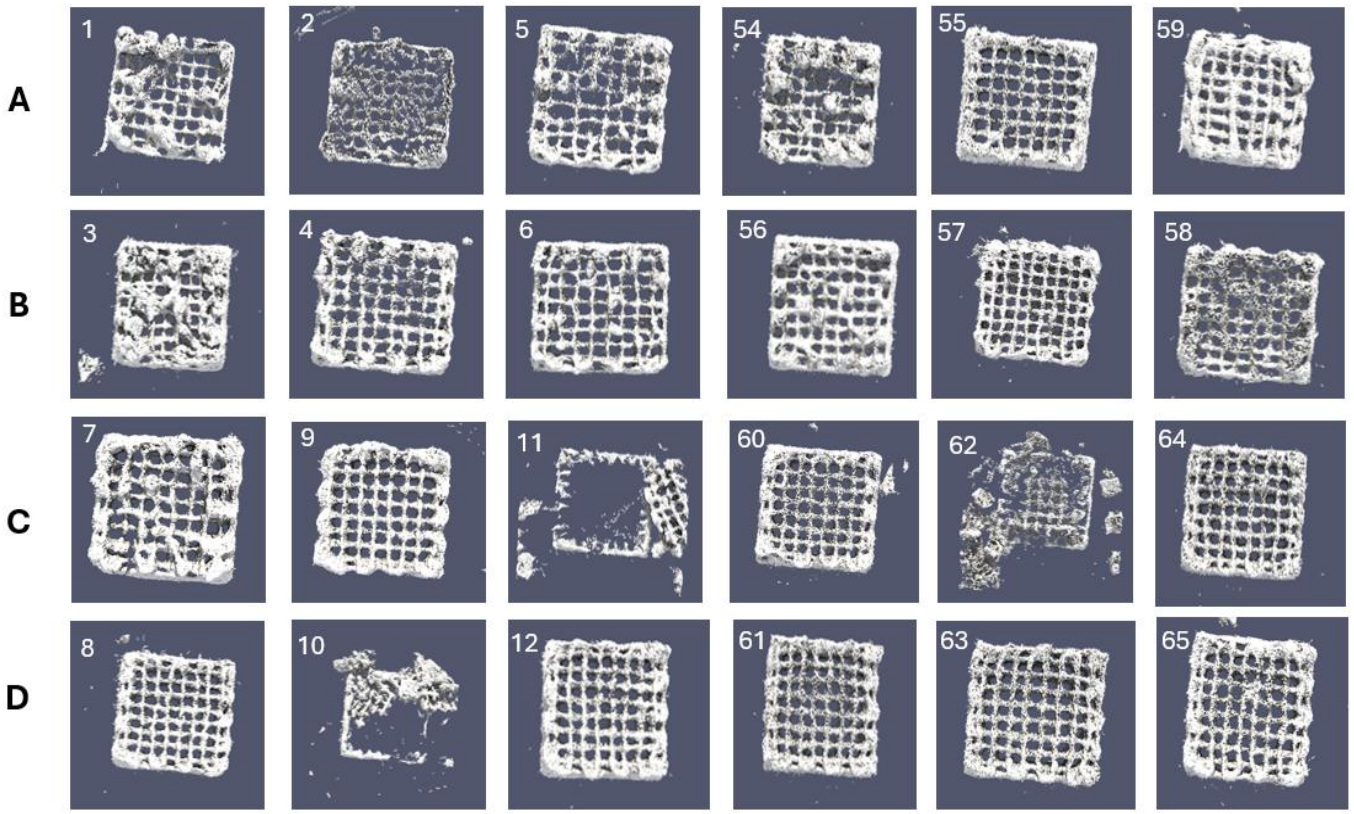

Figure 4.1 An overview of the samples of the present study. (A) Healthy treated, (B) Healthy untreated, (C) OI treated, (D) OI untreated. The numbers correspond to the bioreactors used

Figure 4.2A depicts the total bone volume for OI samples, expressed in mm^3. Limiting to the OI samples, the treated group (n=4 after the exclusions) show slightly increased values in bone volume (mm^3). Indeed, after six weeks, the OI treated group had an average bone volume equal to 60.35 mm^3, while the untreated group (n=5 after the exclusions) had an average bone volume

equal to 58.40 mm^3. However, this difference was not statistically significant. It is also worth noting that the treated group had a higher variability compared to the untreated group. The comparison between treated and untreated groups was also conducted for the healthy samples, as shown in Figure 4.2C. Notably, healthy treated samples (n=4 after the exclusions) had an average bone volume equal to 38.38 mm^3 while the untreated group (n=4 after the exclusions) had an average bone volume equal to 45.13 mm^3. Nevertheless, this difference was again not statistically significant.

Previous trends can be also observed from the examination of the Tissue Mineral Density (TMD), expressed in mg of hydroxyapatite (HA) per cm^3 of the samples. Limiting to OI organoids, Figure 4.2B shows that the treated group had an average TMD of 113.54 mg HA/cm^3, whereas the untreated group had an average TMD of 111.07 mg HA/cm^3. Again, the effect of Teriparatide on TMD was also investigated for healthy samples. Figure 4.2D shows that healthy treated group had an average TMD of 111.76 mg HA/cm^3 while the healthy untreated group had an average TMD of 117.43 mg HA/cm^3. However, the difference was not statistically significant.

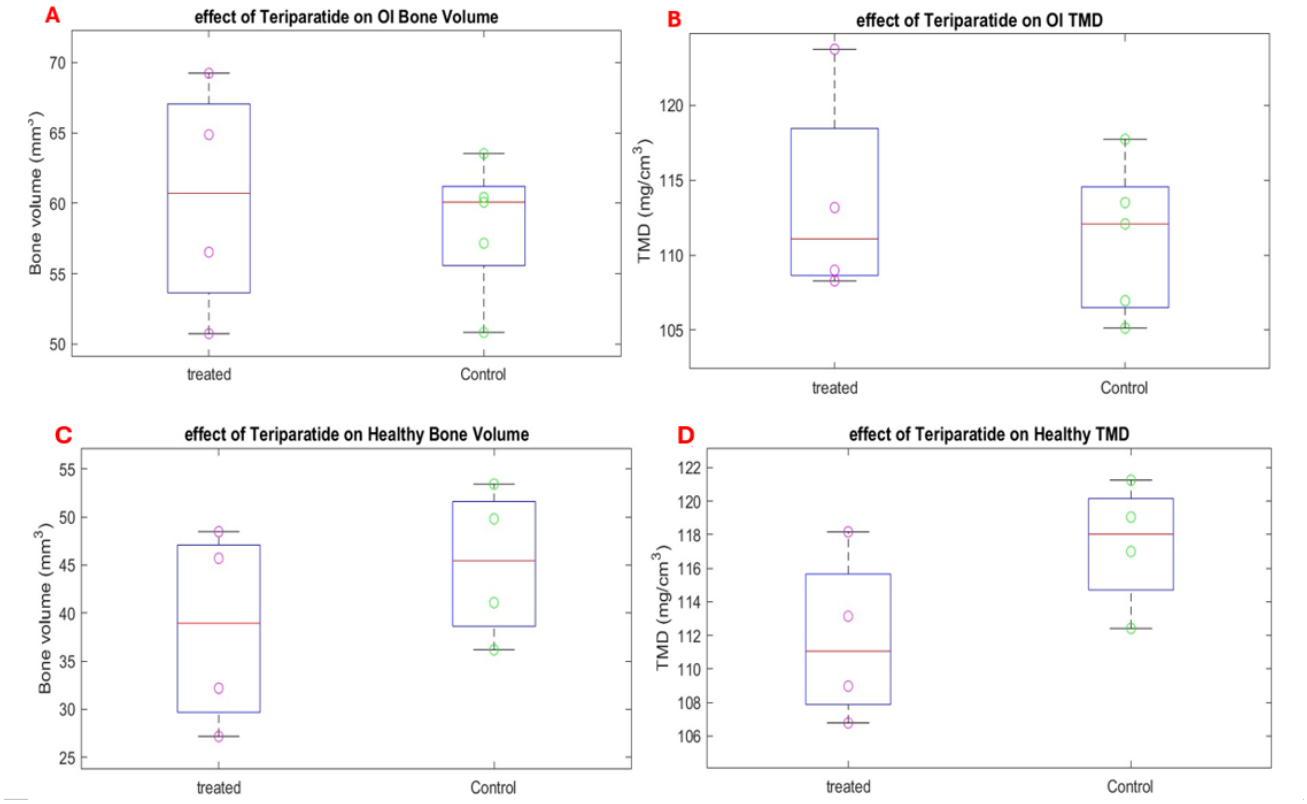

Figure 4.2 (A) Bone volume (mm^3) of Teriparatide treated and untreated OI groups, (B) Tissue Mineral Density (TMD, mg HA/cm^3) of Teriparatide treated and untreated OI groups, (C) Bone volume (mm^3) of Teriparatide treated and untreated healthy groups, (D) Tissue Mineral Density (TMD, mg HA/cm^3) of Teriparatide treated and untreated healthy groups.

Moreover, the mineralization parameters of OI untreated and healthy untreated groups were compared. For the sake of clarity, healthy untreated group had an average bone volume equal to 45.13 mm^3, while OI untreated group had an average bone volume equal to 58.40 mm^3. Healthy untreated group had an average TMD of 117.43 mg HA/cm^3, whereas OI untreated group had an average TMD of 111.07 mg HA/cm^3. Notably, OI showed statistically significantly higher values of Bone Volume, while lower values of Tissue Mineral Density.

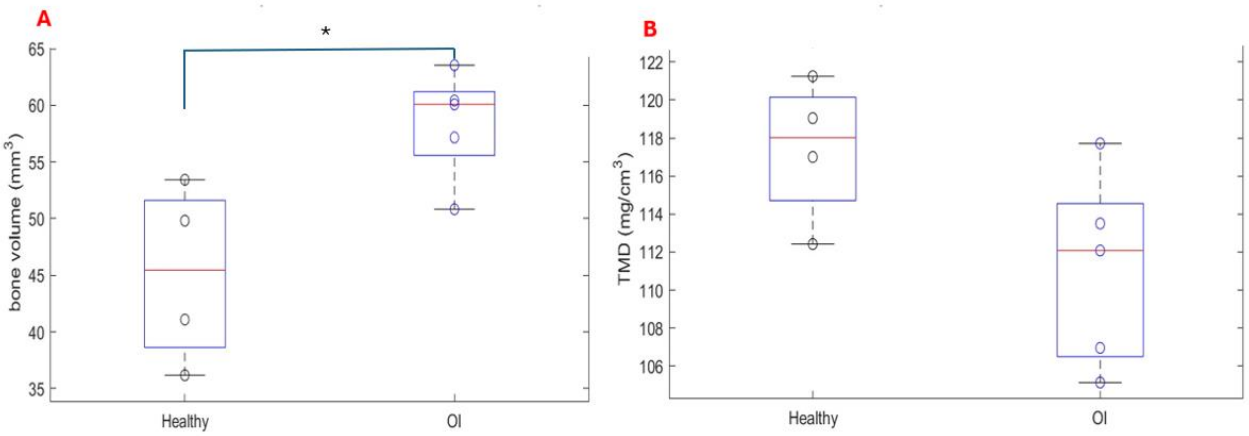

Figure 4.3 (A) Bone volume (mm^3) of healthy untreated and OI untreated groups. (B) Tissue Mineral Density (TMD, mg HA/cm^3) of healthy untreated and OI untreated groups

4.2 Mechanical Loading- Stiffness Analysis

The data from the mechanical stimulation unit, acquired during mechanical loading, were analysed with a pre-existing phyton script and then processed with OriginLab. The present analysis focused in particular on dynamic stiffness (N/mm) data. Besides the discarded samples, some other measurements were not included, due to specific technical issues. Likely, during these tests the piston was touching the walls of the external cylinder and then it resulted in extremely high values.

Regarding OI samples, both treated and untreated (Figure 4.4A), results showed an initial decrease in dynamic stiffness, which can be explained by the dissolving of excess gelatin. Then, both groups showed an increase in this parameter, which can be reasonably motivated by the mineralization process. Notably, on day 43, the end time point, the treated group had a normalized stiffness equal to 256%, while the untreated group had a normalized stiffness equal to 210%. However, this difference was not statistically significant (p=0.07).

Focusing on the healthy group (Figure 4.4B), the same trend (decrease and then increase) could be observed, as these effects are independent of the cell donor. Concerning the end time point, the healthy treated group had a normalized stiffness value equal to 214%, while the healthy untreated group had a normalized stiffness value equal to 247%. However, this difference was again not statistically significant (p=0.31).

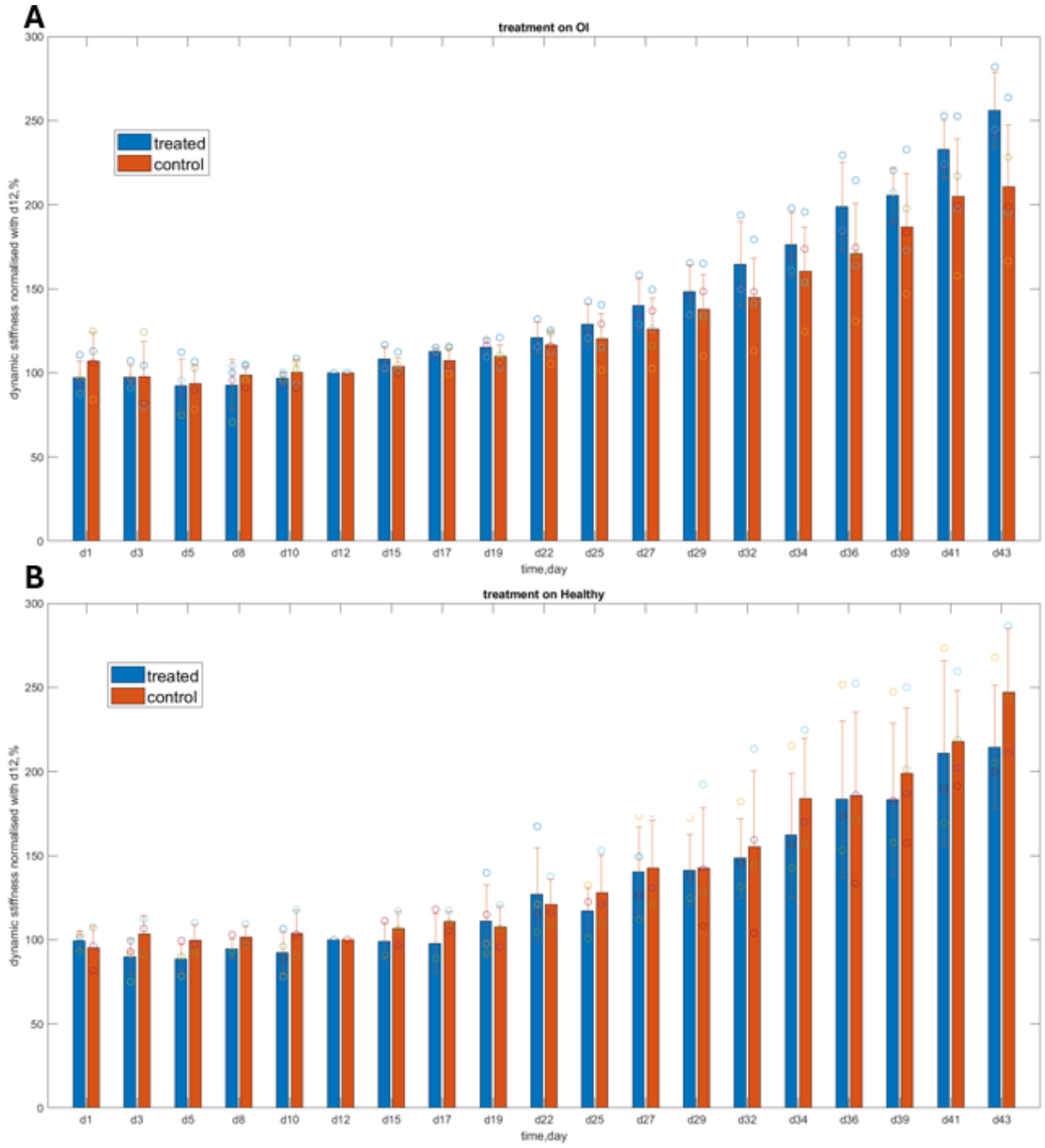

Figure 4.4 Dynamic Stiffness of OI bone organoids (A) and of healthy bone organoids (B)

5 Discussion

In my previous project, MAD1, almost the half of the printed bone organoids could not reach the fifth week of culture. This issue was mainly due to the adoption of black sample holders, which were too high. Indeed, for this reason the piston probably hit the samples in an uncontrolled manner during the handling for μCT scans, medium change and mechanical loading. Then for the present study, MAD2, those black sample holders were replaced with white shorter ones. Both sample holders can be seen in Figure 5.1.

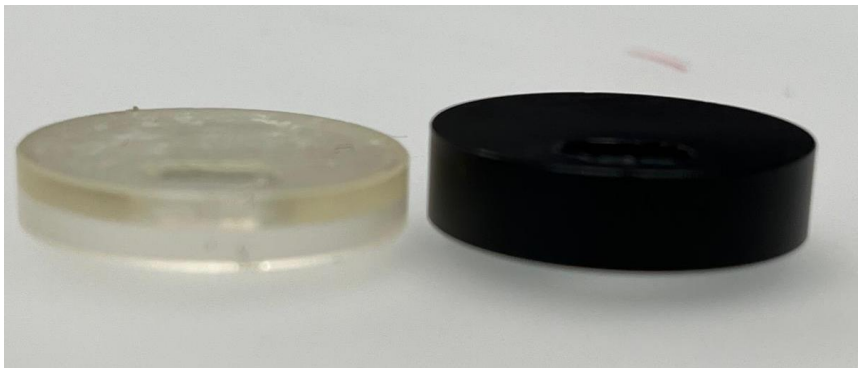

Figure 5.1 The different sample holders

In this way, the samples were lower, and the piston could not hit them uncontrollably. However, changing the sample holders was not an action without collateral effects. Indeed, being lower, the samples could not be entirely detected in the weekly μCT scans. Unfortunately, we discovered this issue only during week 5 of culture. Before the last scan, the number of slices was increased from 120 to 170, including the entire height of the samples. Hence, only the scans performed on week 6 were fine. The images resulting from these scans were further converted into 3D-renderings, helping in the choice of which samples deserved to be kept for the analysis and which not.

In this project, the effect of Teriparatide on human cells-derived 3D bioprinted bone organoids was studied. Teriparatide is a promising anabolic drug, which is already approved for osteoporosis management and for this reason it has been also tested on OI adult patients. Its pharmacokinetics was deeply investigated in a study [24] which showed that the peak concentration in plasma was around 700 pg/mL. Considering the molecular weight of Teriparatide, which is 4117.77 g/mol, this translates in a peak concentration in plasma equal to 0.17 nM. Moreover, their results also showed that the drug can be cleared by the human body in about 2 hours. Based on these interesting data, in the present study we tested Teriparatide

using a concentration equal to 0.2 nM, and two hours after the administration, medium was changed. Regarding OI-specific organoid, the drug seems to have only a limited effect on the average values of Bone Volume or Tissue Mineral Density from week 6. This fact was not a great surprise, since they were in line with a student project within the Laboratory of Bone Biomechanics [25]. However, in that study, normalizing with mineral values of week-2, before the drug study started, they were able to show more appreciable differences between treated and untreated samples. Unfortunately, we were not able to perform the same normalization, since we did not have the mineralization values from week-2. On the other hand, a limited efficacy of this drug on the specific OI type (III/IV) would be not so surprising, since a clinical trial [18] already showed that patients affected with this type showed no benefits in terms of Bone Mineral Density after 18 months of treatment with Teriparatide. Considering their results, teriparatide was suggested only for the treatment of adult type I OI. Even the effects in the treatment of atypical femoral fractures were questioned, since they inevitably lack a proper control to prove the role of teriparatide in bone healing.
Regarding the healthy samples, we observed a slightly negative effect of the drug on the average values of mineralization parameters. Even though the differences were not pronounced, this fact was surprising. However, such differences could be also explained by the huge heterogeneity of printing quality. Indeed, healthy treated samples were worse printed compared to healthy untreated, as can be easily seen in Figure 4.1.

For what concerns dynamic stiffness data, these were more of interest. Focusing on OI group, it was visible an effect of teriparatide from the beginning of drug treatment (day 12), which continued over the entire culture period. On the end time point there was a notable, but not statistically significant ($p=0.07$), difference between the treated and untreated group. Then, it could be claimed that teriparatide could have a role on stimulating cells to produce collagen matrix, improving mechanical properties and then strength of OI organoids. This is a consideration that is also supported from Jacob's study at the LBB [25]. Then, this could be an indication in OI management, since it is reasonable that the drug could improve bone strength in OI patients, even without increasing BMD. Regarding the healthy cohort, the untreated group showed higher values compared to the treated group. Once again, this surprising fact could be attributable to the printing quality.

Moreover, it is also worth mentioning that the adopted mechanical loading protocol did not achieve its original aim. Indeed, the starting idea was that the loading should result in fractures for the OI untreated group, while not breaking the OI treated samples. As reported, the broken samples number 10 and 11 are just the result of technical issues such as the fall of the piston which crashed on them. This situation could be due to many causes. First, the mechanical loading was not always properly performed, since with the white sample holders, the samples were likely too low, and the piston could not go all the way down to perform the loading. On the other side, the parameters of loading were probably just not enough high to break the samples. Lastly, it is also worth saying that none of the healthy samples broke during the culture.

6 Conclusion

In this study, we were able to show an effect of teriparatide in increasing the dynamic stiffness of osteogenesis imperfecta patient-derived organotypic bone models. On the other side, we could not fully demonstrate, in the same samples, an effect of the drug in mineralization parameters such as Bone Volume and Tissue Mineral Density. Regarding the healthy group, teriparatide seemed to have a counterintuitive negative effect on the mineral values. Moreover, only limited evidence of hypermineralization, a typical OI feature, was detected. However, these unexpected results could be attributable to a suboptimal printing quality of healthy group, due to too low room temperature values during printing day. Future studies should focus on the understanding of the influence of room temperature on the bioprinting process. Moreover, further analysis should investigate the medium levels of bone formation markers, such as procollagen propeptides or sclerostin. Lastly, RNA analysis could be useful in the elucidation of molecular aspects underlying OI disease and its response to drug treatment.

7 References

[1] Marini, J.C., Forlino, A., Bächinger, H.P., Bishop, N.J., et al., Osteogenesis imperfecta. *Nat Rev Dis Primers* 2017, *3*, 1–19.

[2] Boyde, A., Travers, R., Glorieux, F.H., Jones, S.J., The mineralization density of iliac crest bone from children with osteogenesis imperfecta. *Calcif Tissue Int* 1999, *64*, 185–190.

[3] Bishop, N., Bone Material Properties in Osteogenesis Imperfecta. *Journal of Bone and Mineral Research* 2016, *31*, 699–708.

[4] Ralston, S.H., Gaston, M.S., Management of Osteogenesis Imperfecta. *Front Endocrinol (Lausanne)* 2020, *10*, 924.

[5] Tauer, J.T., Abdullah, S., Rauch, F., Effect of Anti-TGF-β Treatment in a Mouse Model of Severe Osteogenesis Imperfecta. *Journal of Bone and Mineral Research* 2019, *34*, 207–214.

[6] Akiva, A., Melke, J., Ansari, S., Liv, N., et al., An Organoid for Woven Bone. *Advanced Functional Materials* 2021, *31*, 2010524.

[7] Zhang, J., Wehrle, E., Rubert, M., Müller, R., 3D Bioprinting of Human Tissues: Biofabrication, Bioinks, and Bioreactors. *IJMS* 2021, *22*, 3971.

[8] Zhang, J., Eyisoylu, H., Qin, X.-H., Rubert, M., et al., 3D bioprinting of graphene oxide-incorporated cell-laden bone mimicking scaffolds for promoting scaffold fidelity, osteogenic differentiation and mineralization. *Acta Biomaterialia* 2021, *121*, 637–652.

[9] Zhang, J., Wehrle, E., Adamek, P., Paul, G.R., et al., Optimization of mechanical stiffness and cell density of 3D bioprinted cell-laden scaffolds improves extracellular matrix mineralization and cellular organization for bone tissue engineering. *Acta Biomaterialia* 2020, *114*, 307–322.

[10] Zhang, J., Griesbach, J., Ganeyev, M., Zehnder, A.-K., et al., Long-term mechanical loading is required for the formation of 3D bioprinted functional osteocyte bone organoids. *Biofabrication* 2022, *14*, 035018.

[11] de Leeuw, A.M., Graf, R., Lim, P.J., Zhang, J., et al., Physiological cell bioprinting density in human bone-derived cell-laden scaffolds enhances matrix mineralization rate and stiffness under dynamic loading. *Front. Bioeng. Biotechnol.* 2024, *12*.

[12] Griesbach, J., Leeuw, A. de, Minacci, T., Kodiyan, B., et al., 3D-bioprinted patient-specific organotypic bone model mimicking mineralization dysregulation in FKBP10-related osteogenesis imperfecta. 2024, 2024.05.20.594917.

[13] Quattrocchi, E., Kourlas, H., Teriparatide: A review. *Clinical Therapeutics* 2004, *26*, 841–854.

[14] Liu, W., Nicol, L., Orwoll, E., Current and Developing Pharmacologic Agents for Improving Skeletal Health in Adults with Osteogenesis Imperfecta. *Calcif Tissue Int* 2024.

[15] Uveges, T.E., Kozloff, K.M., Ty, J.M., Ledgard, F., et al., Alendronate treatment of the brtl osteogenesis imperfecta mouse improves femoral geometry and load response before fracture but decreases predicted material properties and has detrimental effects on osteoblasts and bone formation. *J Bone Miner Res* 2009, *24*, 849–859.

[16] Leali, P.T., Balsano, M., Maestretti, G., Brusoni, M., et al., Efficacy of teriparatide vs neridronate in adults with osteogenesis imperfecta type I: a prospective randomized international clinical study. *Clin Cases Miner Bone Metab* 2017, *14*, 153–156.

[17] Gatti, D., Rossini, M., Viapiana, O., Povino, M.R., et al., Teriparatide Treatment in Adult Patients with Osteogenesis Imperfecta Type I. *Calcif Tissue Int* 2013, *93*, 448–452.

[18] Orwoll, E.S., Shapiro, J., Veith, S., Wang, Y., et al., Evaluation of teriparatide treatment in adults with osteogenesis imperfecta. *J Clin Invest* 2014, *124*, 491–498.
[19] Hald, J.D., Keerie, C., Weir, C.J., Javaid, M.K., et al., Protocol of a randomised trial of teriparatide followed by zoledronic acid to reduce fracture risk in adults with osteogenesis imperfecta. *BMJ Open* 2023, *13*, e078164.
[20] Aspenberg, P., Genant, H.K., Johansson, T., Nino, A.J., et al., Teriparatide for acceleration of fracture repair in humans: A prospective, randomized, doubleblind study of 102 postmenopausal women with distal radial fractures. n.d.
[21] Holm, J., Eiken, P., Hyldstrup, L., Jensen, J.-E.B., Atypical Femoral Fracture in an Osteogenesis Imperfecta Patient Successfully Treated with Teriparatide. *Endocrine Practice* 2014, *20*, e187–e190.
[22] Etxebarria-Foronda, I., An atypical fracture in male patient with osteogenesis imperfecta. *Ccmbm* 2015.
[23] Tan, J.Y., Seow, C.J., Management of atypical femoral fracture in a patient with osteogenesis imperfecta. *BMJ Case Reports* 2017, bcr-2017-221835.
[24] Hämmerle, S.P., Mindeholm, L., Launonen, A., Kiese, B., et al., The single dose pharmacokinetic profile of a novel oral human parathyroid hormone formulation in healthy postmenopausal women. *Bone* 2012, *50*, 965–973.
[25] Jacobs, N., Personalized bone organoids for drug efficacy testing in children with osteogenesis imperfecta. 2023.

8 Appendix

Here are presented some results of my previous study (MAD1), which was performed exactly as MAD2 apart from two differences. The first is that in MAD1 we used a cell concentration of 5 million per mL of ink. The second is that the culture period lasted only five weeks. In Figure 8.1 it is possible to see the Tissue Mineral Density, expressed in mg HA/cm^3, of every group. It can be observed an effect of the drug treatment on OI samples, at least considering the average values. In the first two weeks TMD is comparable, while starting from the third week (when drug treatment started), the mineralization of the treated group started to increase faster than the untreated one. On the other hand, such difference could be not detected in the healthy samples, and, surprisingly, the treated group had lower values than the untreated group.

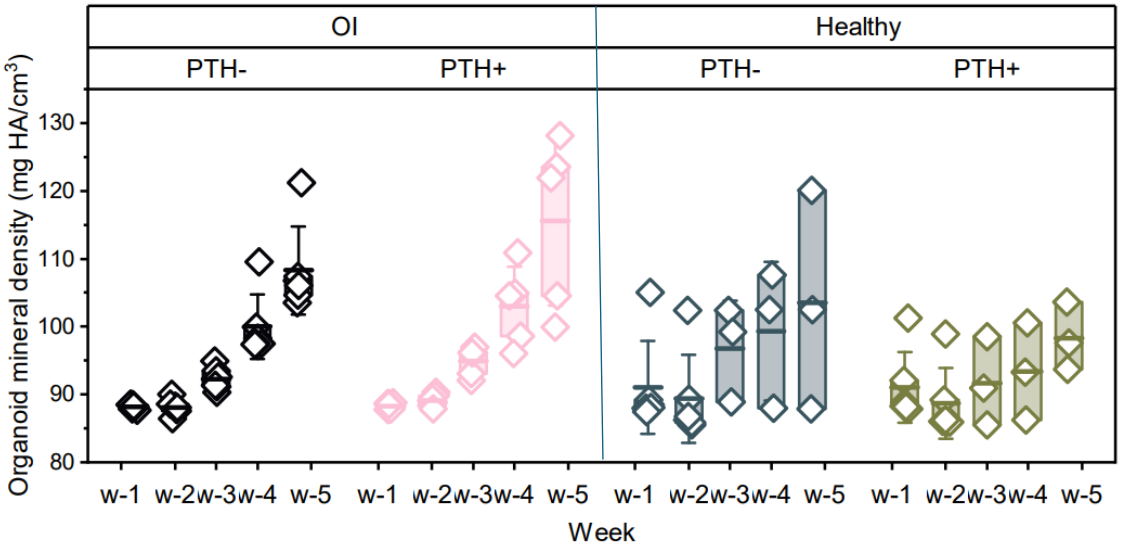

Figure 8.1 Tissue Mineral Density (TMD, mg HA/cm^3) of Teriparatide treated and untreated OI and healthy groups