# Supplementary information for

## A microphysiological system for studying human bone biology under simultaneous control of oxygen tension and mechanical loading

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This file includes: Fig. S1 to S8

Tab. S1 to S4

Supporting text (Fig. S3 and S8)

Supplementary information references

Other supporting information for this manuscript include the following:

| | |
|---|---|
| Supplementary videos: | video_S1_normoxia_no-load.avi |
| | video_S2_normoxia_load.avi |
| | video_S3_physoxia_no-load.avi |
| | video_S4_physoxia_load.avi |
| Supplementary scripts/codes: | user_interface.txt |
| | oxygen_control_loop_p_element.txt |
| | mechanical_loading.txt |

**A** *3D printed components*

| | Name | Printer | Material | Comments |
|---|---|---|---|---|
| a | Waste container lid | Ultimaker 3 extended | Polypropylen | 0.1 mm layer height, autoclavable |
| b | Waste container | Ultimaker 3 extended | Polypropylen | 0.1 mm layer height, autoclavable |
| c | Reservoir RII lid | Ultimaker 3 extended | Polypropylen | 0.1 mm layer height, PVAsupport, autoclavable |
| d | Reservoir RII | Ultimaker 3 extended | Polypropylen | 0.1 mm layer height, autoclavable |
| e | Tube connector | Formlabs 3B | Surgical Guide SD resin | 0.05 mm layer height, UV cured, autoclavable |
| f | Holder plate for all components of the MPS | Prusa MKII and Ultimaker 3 extended | PLA | 0.2 mm layer height |
| g | Modified lid for MPS | Formlabs 3B | Surgical Guide SD resin | 0.05 mm layer height, UV cured, autoclavable |
| h | Threaded piston for adjusting strain on sensor | Formlabs 3B | Surgical Guide SD resin | 0.05 mm layer height, UV cured, autoclavable |
| i | LUER manifold | Formlabs 3B | Surgical Guide SD resin | 0.05 mm layer height, UV cured, autoclavable |

**A'** *Complete MPS setup for individual control of mechanical loading and oxygen tension.*

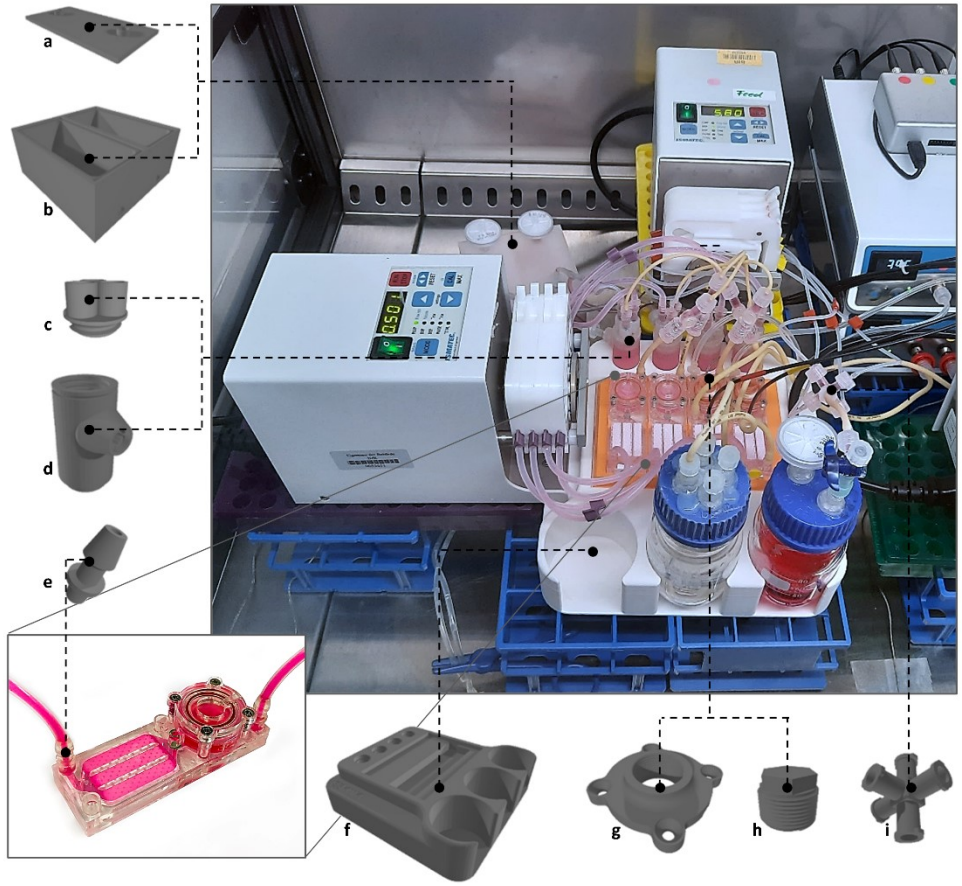
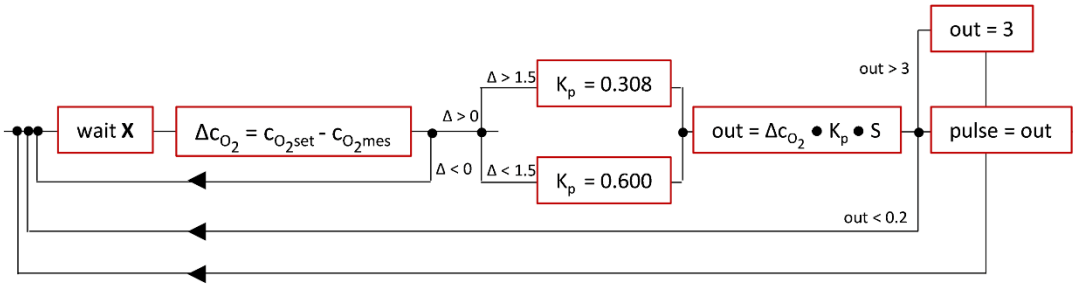



**Fig. S1**: **Complete assembly of the MPS for individual control of mechanical load and oxygen tension including all self-designed and manufactured 3D-printed components**
**(A)** Overview of all 3D printed components with information on the employed printers and printing materials. **(A')** Complete setup for the individual control of mechanical loading and oxygen tension in four MPS main units in parallel. The localisation of all self-designed 3D-printed components in the system is indicated

**A**

| Parameter | Value (± standard deviation) |
|---|---|
| **Response time** | 6.93 s (± 0.83 s) |
| **Reaction time** | 60 s |
| **Minimum pulse length** | 0.2 s |
| **Maximum pulse length** | 3.0 s |
| **Proportionality factor** | 0.308 [% $O_2$ change/1 s pulse length] |

**B**




| | |
|---|---|
| S | safety factor (gain, prevents overshoot of exchange gas) |
| $c_{O_2set}$ | target value (from input) |
| $c_{O_2mes}$ | actual value (from sensor) |
| $K_p$ | proportionality factor (derived from real world data, might be changed when target is approached) |
| X | reaction time [s], accounts for system inertia |

**Fig. S2: Oxygen control loop**
**(A, B)** The Kp-value and other system parameters shown in table **(A)** were used to set up a **(B)** conditional p-element based control loop for feed-back control of oxygen tension in the fluid circuit of the MPS.

***Fig. S3:*** *Controlled hypoxic conditions in MPS lead to expression of hypoxia-inducible factor (Hif) regulated luciferase-reporter construct by U2OS-HRE-LUC cells. Response of U2OS-HRE-LUC cells to different oxygen levels in the MPS*

*Method -Testing oxygen conditions with U2OS-HRE-LUC cells in the MPS*

U2OS-HRE-LUC line (kindly provided by Professor Margaret Ashcroft) is a human osteosarcoma cell line (U2OS) stably expressing a triple tandem repeat of a hypoxia response element (HRE) fused to a luciferase reporter gene (LUC). In response to hypoxia, hypoxia-inducible factor-1 (Hif-1), a transcriptional complex of stabilised Hif-1α and Hif-1β, binds to the HRE, leading to expression of the luciferase reporter gene. Under normoxic conditions, Hif-1α is mainly regulated by protein stability and rapidly degraded by the proteasome (1). $1.25 \times 10^5$ U2OS-HRE-LUC cells were seeded onto gelatine-precoated coverslips (10 mm × 10 mm) and maintained for two days in U2OS medium (DMEM-HG supplemented with 10 % FBS, 100 U $mL^{-1}$ penicillin,100 µg $mL^{-1}$ streptomycin and 1 mM sodium-pyruvate) under standard culture conditions (37 °C, 5 % $CO_2$, 95 % humidity) to allow formation of a confluent monolayer. Each coverslip was then placed on its own MPS holder plate and transferred to a MPS culture chamber containing U2OS-MPS-medium (DMEM-HG supplemented with 5 % FBS, 1 mM sodium-pyrovate, 100 U $mL^{-1}$ penicillin, 100 µg $mL^{-1}$ streptomycin). Four different oxygen conditions were tested in a single run. The oxygen concentration was adjusted to 1 % (hypoxia), 5 % (physoxia), and 19.8 % (normoxia), respectively. As positive control, one unit was exposed to the hypoxia mimetic agent deferoxamine mesylate (DFX) at a concentration of 250 µM under normoxia (19.8 % oxygen level). After 24 h, the coverslips were removed, washed in DPBS (300 µl) and incubated in 400 µl of firefly luciferase substrate buffer (2) (Promega cat.No. E1531) on a rotary shaker (240 rpm) at room temperature (RT) for 10 min. Finally, samples were transferred to a white 96-well microtiter plate in 100 µL triplets and luminescence was measured at 0.5 min integration on a multimode microplate reader (Mitras LB 940, Berthold technologies, Montlucon, France).

*Results*

We investigated the biological response of human bone cells to distinct oxygen tension, which were individually adjusted using the P-element-based regulatory circuit in the MPS main units. For this purpose, human bone osteosarcoma epithelial cells (U2OS-HRE-LUC) carrying a stable luciferase reporter gene construct (LUC) in combination with the hypoxia responsive element (HRE) (1) were seeded on coverslips and transferred to the individual MPS main units. To verify whether the MPS allows effective control of oxygen levels and stimulation of hypoxia-inducible factor (Hif)-1 formation in U2OS-HRE-LUC cells in response to reduced oxygen levels in the MPS, cells were individually subjected to either hypoxia (1 % oxygen), physoxia (5 % oxygen), or normoxia (19.8 % oxygen) for 24 h in their respective MPS main unit. For the proof of concept, U2OS-HRE-LUC cells in the fourth MPS main unit under normoxia were additionally treated with the hypoxia mimetic DFX, as this is known to stimulate the formation of Hif-1 and thus the induction of the stably transfected luciferase reporter gene (1). The highest increase in luciferase activity was obtained in response to hypoxia (1 % oxygen), followed by stimulation of U2OS-HRE-LUC cells with DFX (Fig. S3). No increased luciferase activity was measured under both physoxia and normoxia. The results show that, in our setup, hypoxic conditions can be effectively controlled at a set point value of 1 % oxygen, leading to the stabilization and activation of Hif1 as shown by an increase of luciferase activity in U2OS-HRE-LUC cells, whereas physoxia (5 %) apparently still allows efficient oxygen-dependent degradation of the Hif-1α subunit.

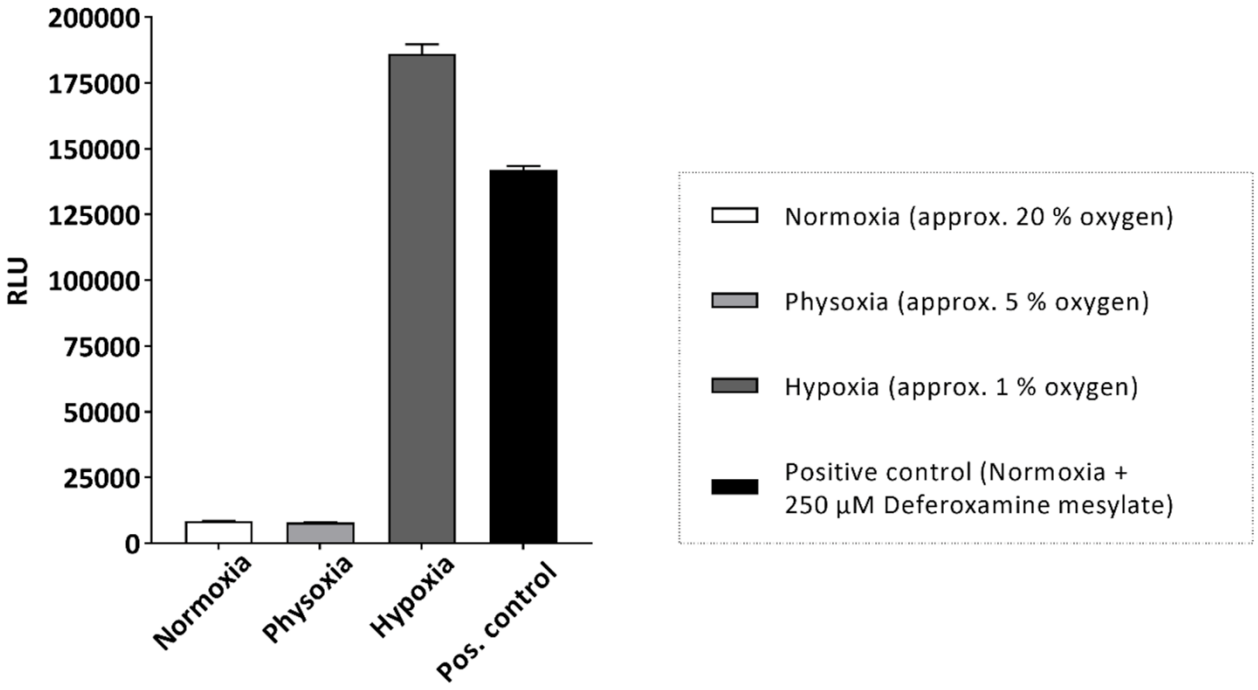



**Fig. S3: Controlled hypoxic conditions in MPS lead to expression of hypoxia-inducible factor (Hif) regulated luciferase-reporter construct by U2OS-HRE-LUC cells.**

$1.25 \times 10^5$ U2OS-HRE-LUC cells were seeded on gelatine-coated coverslips (10 mm × 10 mm) and kept in the MPS for 24 h. The oxygen content of the U2OS-MPS medium was previously adjusted to 1 %, 5 %, and 19.8 %. As a positive control, cells in one main culture unit were treated with 250 µM DFX at an oxygen level of 19.8 % to chemically induce expression of luciferase-reporter construct. The coverslips were then removed from the culture chambers of the MPS main units, washed with PBS, and incubated in firefly luciferase substrate on a rotary shaker (240 rpm) at room temperature for 10 min. For measurement, samples of three times 100-µl were transferred to a white 96-well microtiter plate and luminescence was measured at an integration time of 0.5 min using a microplate reader. Shown is the mean ± standard deviation (SD), n=1, SD corresponds to a technical triplicate.

**A**

$$F = \frac{E * A * \Delta h}{h_0}$$

F = force
E = coefficient of elasticity (young's modulus)
A = scaffold area
$h_0$ = scaffold height
Δh = height change when force is applied
d = diameter of the scaffold

**B**

| Properties of Optimaix 3D type I collagen scaffold | |
|---|---|
| **d [m]** | 0.013 |
| **$h_0$ [m]** | 0.003 |
| **E [kPa]** | 8 |
| **A [m²]** | 0.00013 |
| **Pore size [mm]** | 0.08 |

**C**

| Force needed for 10 % compressive strain | |
|---|---|
| **Compression [%]** | 10 |
| **Δh [m]** | 0.0003 |
| | |
| **F [N]** | 0.10619 |
| **F [mN]** | **106.18583** |

**Fig. S4: Calculation of the force necessary for 10 % compression of the type I collagen scaffold**
**(A)** Formula for calculating the force required for a 10 % compressive load. **(B)** Parameters of the Optimaix 3D type I collagen scaffold. **(C)** Calculated force required for a 10 % compressive strain.

**Tab. S1: Background data of the cancellous bone donors**
For each donor, the internal donor number and the icon used consistently in all figures, the age, sex, type of surgery performed, comorbidities and daily medications are listed.

| Donor | Icon | Sex | Age | Surgery | Comorbidities | Medication |
|---|---|---|---|---|---|---|
| **1** | ○ | male | 77 | right cementless total hip arthroplasty | ASA score: 3; hypothyroidism, bypass, B-cell non Hodgkin's lymphoma + bone metastases, lung resection, coronary artery disease, arterial hypertension, vitamin D deficiency | L-Thyroxine, Beta blocker, Uricostatic, Antihypertensive drug, Cholesterol absorption inhibitor, Statins, Anticonvulsant drug |
| **2** | □ | female | 67 | left cementless total hip arthroplasty | ASA Score: 2; spinal stenosis, obesity, spondyloarthritis, osteochondrosis, essential hypertension, | ACE inhibitor, Statins |
| **3** | △ | male | 55 | right cementless short stem femoral head prosthesis with acetabular prosthesis | ASA score: 1 | |
| **4** | ◇ | female | 67 | left cementless short stem total hip arthroplasty | ASA score: 2; osteoporosis, essential hypertension | Remedy against water retention (Tensoflux), synthetic non-steroidal selective oestrogen receptor modulator (Evista) |

**ASA - American Society of Anaesthesiologists*

**Tab. S2: Confidence intervals and p-values of the statistically analysed data sets for cell viability and aerobic glycolysis**
Confidence intervals and p-values of statistical analyses of the datasets obtained from measurements of **(A)** DNA content, **(B)** protein content, **(C)** mitochondrial activity, **(D, D')** lactate production, **(E, E')** glucose consumption. All analysed raw data were from n=4 donors. All parameters **A–E** were measured under either normoxia or physoxia, and in the presence (load) or absence of mechanical loading. The calculation of confidence intervals and p-values is described in the Experimental. Blue shading indicates effects with a significance level of $p < 0.005$.

**A**

| Effect | Comparison | P-value | △ Confidence interval | | |
|---|---|---|---|---|---|
| | | | LOWER | MEAN | UPPER |
| DNA amount | normoxia vs. physoxia | 0.6900 | -0.71 | 0.16 | 1.03 |
| DNA amount | normoxia vs. normoxia load | 0.3360 | -0.48 | 0.39 | 1.26 |
| DNA amount | physoxia vs. physoxia load | 0.0439 | 0.03 | 0.90 | 1.77 |
| DNA amount | physoxia load vs. Normoxia load | 0.1160 | -0.20 | 0.67 | 1.54 |
| DNA amount | physoxia vs. normoxia load | 0.5610 | -1.10 | -0.23 | 0.64 |
| DNA amount | normoxia vs. physoxia load | 0.0223 | 0.19 | 1.06 | 1.93 |

| | | Confidence interval | | |
|---|---|---|---|---|
| | | LOWER | MEAN | UPPER |
| DNA amount | normoxia | 0.83 | 3.57 | 6.31 |
| DNA amount | normoxia load | 1.22 | 3.96 | 6.70 |
| DNA amount | physoxia | 0.99 | 3.73 | 6.47 |
| DNA amount | physoxia load | 1.89 | 4.63 | 7.37 |

**B**

| Effect | Comparison | P-value | △ Confidence interval | | |
|---|---|---|---|---|---|
| | | | LOWER | MEAN | UPPER |
| protein amount | normoxia vs. physoxia | 0.4560 | -36 | 19 | 75 |
| protein amount | normoxia vs. normoxia load | 0.4200 | -35 | 21 | 76 |
| protein amount | physoxia vs. physoxia load | 0.0818 | -7 | 48 | 104 |
| protein amount | physoxia load vs. normoxia load | 0.0909 | -9 | 47 | 102 |
| protein amount | physoxia vs. normoxia load | 0.9490 | -57 | -2 | 54 |
| protein amount | normoxia vs. physoxia load | 0.0229 | 12 | 67 | 123 |

| | | Confidence interval | | |
|---|---|---|---|---|
| | | LOWER | MEAN | UPPER |
| protein amount | normoxia | 100 | 154 | 209 |
| protein amount | normoxia load | 1.22 | 3.96 | 6.70 |
| protein amount | physoxia | 119 | 173 | 228 |
| protein amount | physoxia load | 167 | 222 | 276 |

**C**

| Effect | Comparison | P-value | △ Confidence interval | | |
|---|---|---|---|---|---|
| | | | LOWER | MEAN | UPPER |
| mitochondrial activity | normoxia vs. physoxia | 0.6390 | -0.96 | -0.17 | 0.62 |
| mitochondrial activity | normoxia vs. normoxia load | 0.6660 | -0.94 | -0.16 | 0.63 |
| mitochondrial activity | physoxia vs. physoxia load | 0.0612 | -1.53 | -0.74 | 0.04 |
| mitochondrial activity | physoxia load vs. normoxia load | 0.0573 | -1.55 | -0.76 | 0.03 |
| mitochondrial activity | physoxia vs. normoxia load | 0.9690 | -0.80 | -0.01 | 0.77 |
| mitochondrial activity | normoxia vs. physoxia load | 0.0276 | -1.70 | -0.91 | -0.13 |

| | | Confidence interval | | |
|---|---|---|---|---|
| | | LOWER | MEAN | UPPER |
| mitochondrial activity | normoxia | 2.05 | 3.75 | 5.45 |
| mitochondrial activity | normoxia load | 1.89 | 3.59 | 5.29 |
| mitochondrial activity | physoxia | 1.88 | 3.58 | 5.28 |
| mitochondrial activity | physoxia load | 1.13 | 2.83 | 4.53 |

**D**

| Effect | Comparison | P-value | △ Confidence interval | | |
|---|---|---|---|---|---|
| | | | LOWER | MEAN | UPPER |
| lactate level | normoxia vs. physoxia | 0.0001 | 0.77 | 1.54 | 2.31 |
| lactate level | normoxia vs. normoxia load | 0.0144 | 0.18 | 0.88 | 1.57 |
| lactate level | physoxia vs. physoxia load | < 0.0001 | 1.03 | 1.78 | 2.54 |
| lactate level | physoxia load vs. Normoxia load | 0.0087 | 0.29 | 1.12 | 1.95 |
| lactate level | physoxia vs. normoxia load | 0.1050 | -1.45 | -0.66 | 0.13 |
| lactate level | normoxia vs. physoxia load | < 0.0001 | 1.93 | 2.66 | 3.40 |

| | | Confidence interval | | |
|---|---|---|---|---|
| | | LOWER | MEAN | UPPER |
| lactate level | normoxia | 3.13 | 4.38 | 5.62 |
| lactate level | normoxia load | 4.61 | 5.92 | 7.22 |
| lactate level | physoxia | 3.99 | 5.25 | 6.52 |
| lactate level | physoxia load | 5.75 | 7.04 | 8.32 |

**D'**

| Effect | Comparison | P-value | △ Confidence interval | | |
|---|---|---|---|---|---|
| | | | LOWER | MEAN | UPPER |
| lactate level/DNA day 7 | normoxia vs. physoxia | 0.5510 | -54 | 20 | 95 |
| lactate level/DNA day 7 | normoxia vs. normoxia load | 0.4540 | -49 | 26 | 100 |
| lactate level/DNA day 7 | physoxia vs. physoxia load | 0.8050 | -83 | -8 | 63 |
| lactate level/DNA day 7 | physoxia load vs. normoxia load | 0.9290 | -71 | 3 | 77 |
| lactate level/DNA day 7 | physoxia vs. normoxia load | 0.8750 | -80 | -5 | 69 |
| lactate level/DNA day 7 | normoxia vs. physoxia load | 0.4050 | -46 | 29 | 103 |

| | | Confidence interval | | |
|---|---|---|---|---|
| | | LOWER | MEAN | UPPER |
| lactate level/DNA day 7 | normoxia | 76 | 147 | 218 |
| lactate level/DNA day 7 | normoxia load | 102 | 173 | 244 |
| lactate level/DNA day 7 | physoxia | 97 | 168 | 238 |
| lactate level/DNA day 7 | physoxia load | 105 | 176 | 247 |

**E**

| Effect | Comparison | P-value | △ Confidence interval | | |
|---|---|---|---|---|---|
| | | | LOWER | MEAN | UPPER |
| glucose level | normoxia vs. physoxia | 0.0099 | -0.56 | -0.32 | -0.08 |
| glucose level | normoxia vs. normoxia load | 0.0056 | -0.57 | -0.33 | -0.10 |
| glucose level | physoxia vs. physoxia load | 0.0002 | -0.72 | -0.47 | -0.22 |
| glucose level | physoxia load vs. normoxia load | 0.0002 | -0.74 | -0.49 | -0.24 |
| glucose level | physoxia vs. normoxia load | 0.8890 | -0.25 | -0.02 | 0.22 |
| glucose level | normoxia vs. physoxia load | < 0.0001 | -1.05 | -0.81 | -0.56 |

| | | Confidence interval | | |
|---|---|---|---|---|
| | | LOWER | MEAN | UPPER |
| glucose level | normoxia | 2.48 | 4.88 | 7.28 |
| glucose level | normoxia load | 2.16 | 4.57 | 6.97 |
| glucose level | physoxia | 2.15 | 4.55 | 6.95 |
| glucose level | physoxia load | 1.67 | 4.08 | 6.48 |

**E'**

| Effect | Comparison | P-value | △ Confidence interval | | |
|---|---|---|---|---|---|
| | | | LOWER | MEAN | UPPER |
| glucose level/DNA day 7 | normoxia vs. physoxia | 0.1220 | -86 | -37 | 12 |
| glucose level/DNA day 7 | normoxia vs. normoxia load | 0.9290 | -47 | 2 | 51 |
| glucose level/DNA day 7 | physoxia vs. physoxia load | 0.2380 | -77 | -28 | 22 |
| glucose level/DNA day 7 | physoxia load vs. normoxia load | 0.0134 | -116 | -67 | -17 |
| glucose level/DNA day 7 | physoxia vs. normoxia load | 0.1050 | -88 | -39 | 10 |
| glucose level/DNA day 7 | normoxia vs. physoxia load | 0.0156 | -114 | -65 | -16 |

| | | Confidence interval | | |
|---|---|---|---|---|
| | | LOWER | MEAN | UPPER |
| glucose level/DNA day 7 | normoxia | 56 | 188 | 319 |
| glucose level/DNA day 7 | normoxia load | 58 | 190 | 321 |
| glucose level/DNA day 7 | physoxia | 19 | 151 | 282 |
| glucose level/DNA day 7 | physoxia load | -8 | 123 | 254 |

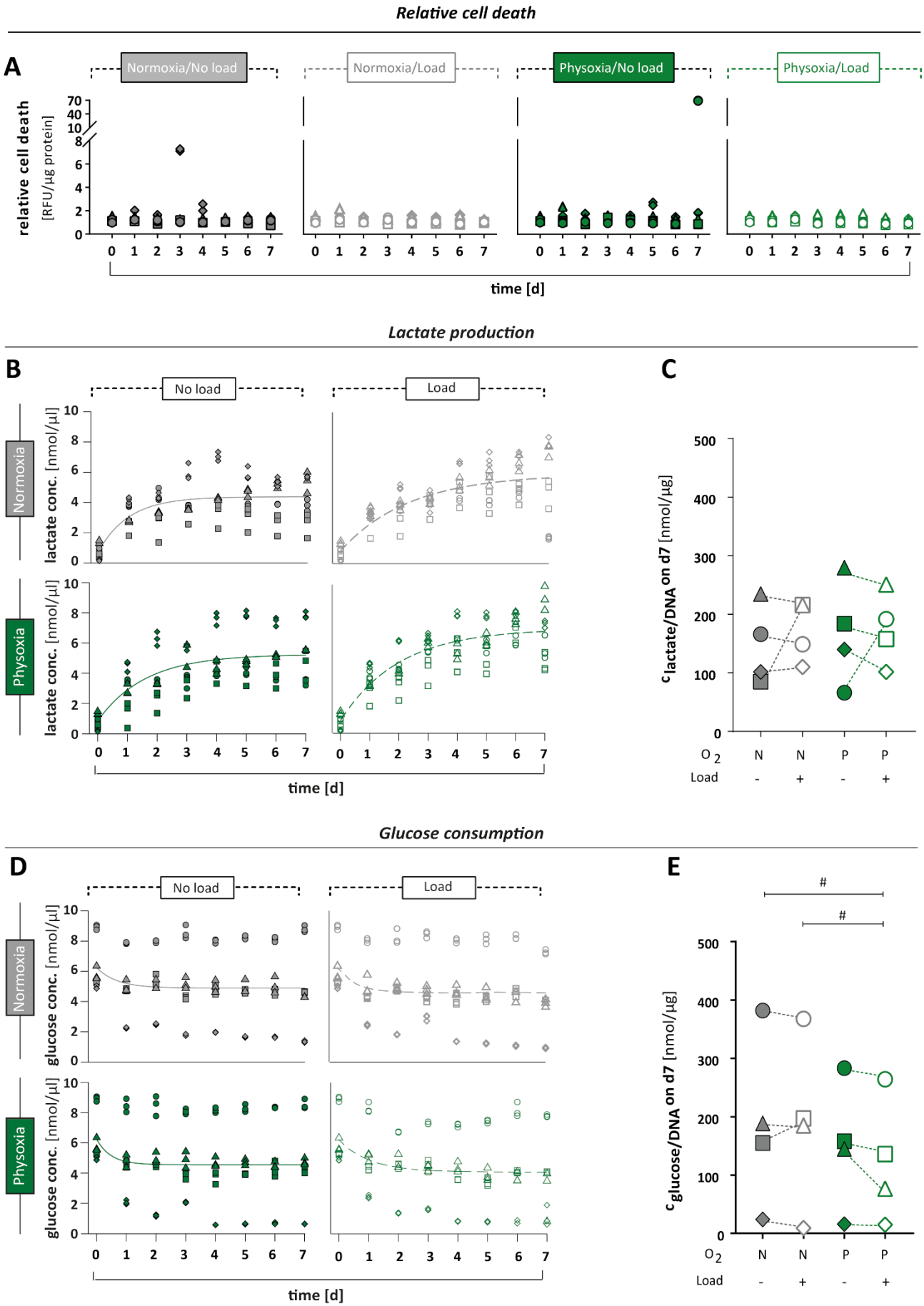

Relative cell death
A
Normoxia/No load
Normoxia/Load
Physoxia/No load
Physoxia/Load
relative cell death
[RFU/µg protein]
time [d]
Lactate production
B
No load
Load
Normoxia
Physoxia
lactate conc. [nmol/µl]
time [d]
C
c lactate/DNA on d7 [nmol/µg]
O2
Load
Glucose consumption
D
glucose conc. [nmol/µl]
E
c glucose/DNA on d7 [nmol/µg]
Donor 1○, 2□, 3△, 4◇
N: Normoxia
P: Physoxia
/ + /□ Load
/ - /■ No load

**Fig. S5: Parameters from culture medium on day seven normalised to the cell density**
(A) Relative cell death assessed by specific protease activity in daily collected samples of MPS medium over seven days, normalized to the total protein content and plotted as single value graphs. (B) Lactate production and (D) glucose consumption was measured in daily medium samples over seven days for all culture conditions. (B + D) Plots of individual data points and asymptotic curves, fitted by a nonlinear mixed model, for all culture conditions (see Experimental section for details). (C) Lactate and **(E)** glucose levels measured in culture medium on day seven and normalised to DNA content. All parameters were measured for the same four donors 1○, 2□, 3△, 4◇ either under normoxia (N) or physoxia (P), with (+/o) or without (-/n) mechanical load. Confidence intervals and p-values obtained by nonlinear mixed model analyses are given in Tab. S2D, D' – E, E'. P values < 0.005 were considered as statistically significant. Different significance levels are indicated as: # $p<0.05$; * $p<0.005$; ** $p<0.0005$; *** $p<0.0001$.

**Tab. S3: Confidence intervals and p-values of the statistically analysed data sets for osteogenic parameters**

Confidence intervals and p-values of statistical analyses of the datasets obtained from measurements of **(A)** pNPP consumption and **(B)** inorganic phosphate release by ALP. All analysed raw data were from n=4 donors. All parameters **A–B** were measured under either normoxia or physoxia, and in the presence (load) or absence of mechanical loading. The calculation of confidence intervals and p-values is described in the Experimental. Blue shading indicates effects with a significance level of $p < 0.005$.

**A**

| Effect | Comparison | P-value | △ Confidence interval | | |
|---|---|---|---|---|---|
| | | | LOWER | MEAN | UPPER |
| alkaline phosphatase | normoxia vs. physoxia | 0.4700 | -14.79 | 7.39 | 29.58 |
| alkaline phosphatase | normoxia vs. normoxia load | 0.4070 | -13.65 | 8.53 | 30.71 |
| alkaline phosphatase | physoxia vs. physoxia load | 0.1630 | -37.07 | -14.88 | 7.30 |
| alkaline phosphatase | physoxia load vs. Normoxia load | 0.1370 | -38.21 | -16.02 | 6.16 |
| alkaline phosphatase | physoxia vs. normoxia load | 0.9100 | -23.32 | -1.14 | 21.05 |
| alkaline phosphatase | normoxia vs. physoxia load | 0.4640 | -29.68 | -7.49 | 14.69 |

| | | Confidence interval | | |
|---|---|---|---|---|
| | | LOWER | MEAN | UPPER |
| alkaline phosphatase | normoxia | 0.51 | 48.26 | 96.00 |
| alkaline phosphatase | normoxia load | 9.04 | 56.79 | 104.53 |
| alkaline phosphatase | physoxia | 7.90 | 55.65 | 103.39 |
| alkaline phosphatase | physoxia load | -6.98 | 40.76 | 88.51 |

**B**

| Effect | Comparison | P-value | △ Confidence interval | | |
|---|---|---|---|---|---|
| | | | LOWER | MEAN | UPPER |
| phosphate level | normoxia vs. physoxia | 0.8200 | -43 | -4 | 35 |
| phosphate level | normoxia vs. normoxia load | 0.0826 | -5 | 33 | 72 |
| phosphate level | physoxia vs. physoxia load | 0.2100 | -62 | -23 | 16 |
| phosphate level | physoxia load vs. normoxia load | 0.0064 | -99 | -61 | -22 |
| phosphate level | physoxia vs. normoxia load | 0.0566 | -76 | -37 | 1.3 |
| phosphate level | normoxia vs. physoxia load | 0.1480 | -66 | -27 | 12 |

| | | Confidence interval | | |
|---|---|---|---|---|
| | | LOWER | MEAN | UPPER |
| phosphate level | normoxia | 109 | 155 | 201 |
| phosphate level | normoxia load | 143 | 189 | 234 |
| phosphate level | physoxia | 105 | 151 | 197 |
| phosphate level | physoxia load | 82 | 128 | 174 |

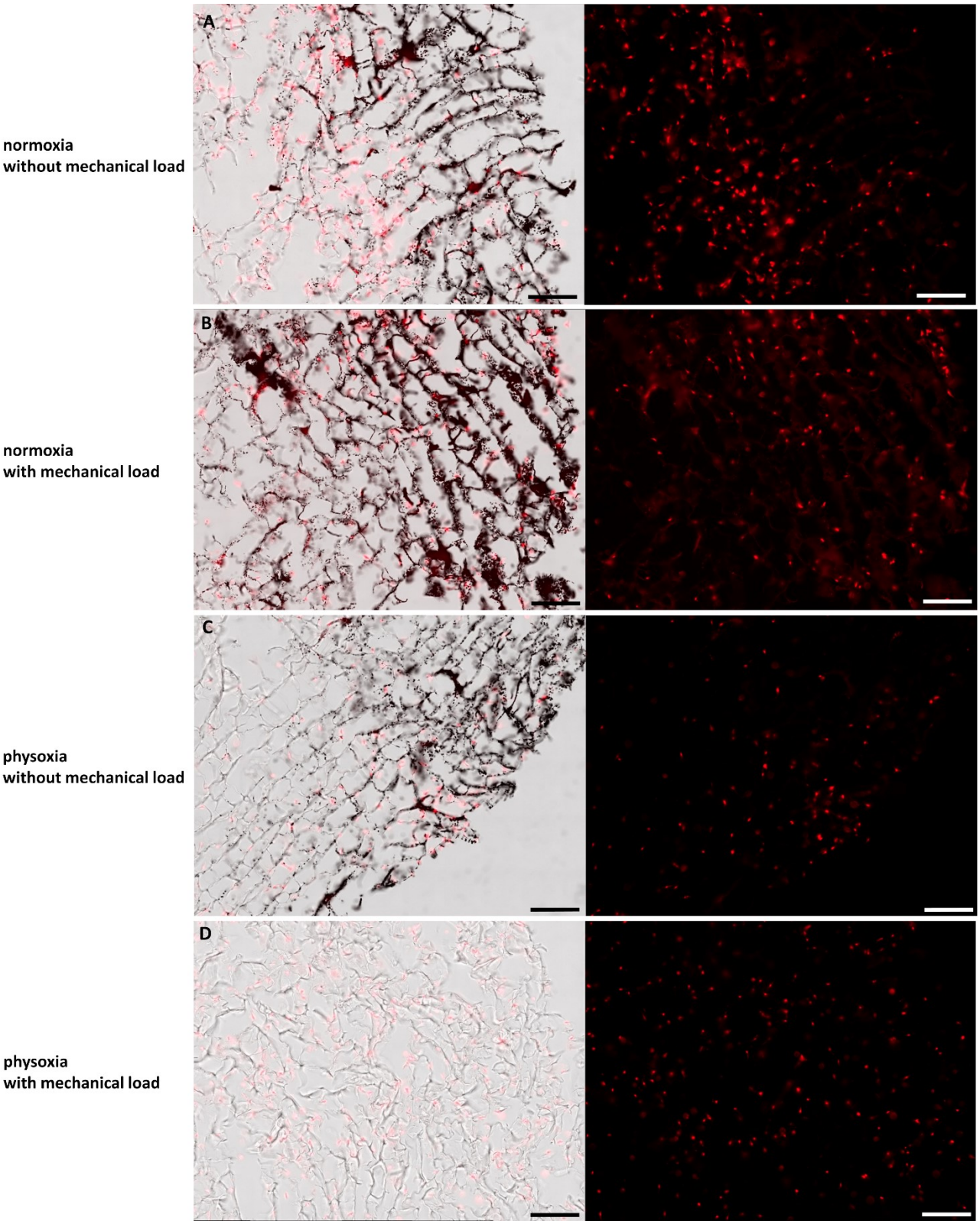



**Fig. S6: Scaffold mineralisation and cell nuclei visualised by von Kossa and DNA staining after seven days of incubation in the MPS.** Selected regions in the (mineralised) periphery of cultivated type I collagen scaffolds (50-µm-sections, donor 3) were imaged as tile scans at 40×-magnification. Right: DRAQ5 fluorescence signals indicate the cell nuclei. Left: Overlay images of fluorescence and brightfield images show the mineralisation-induced black precipitates of the von Kossa staining. Scale bars, 200 µm. For low-magnification overview images of the scaffolds, see Fig. 5 D-G.

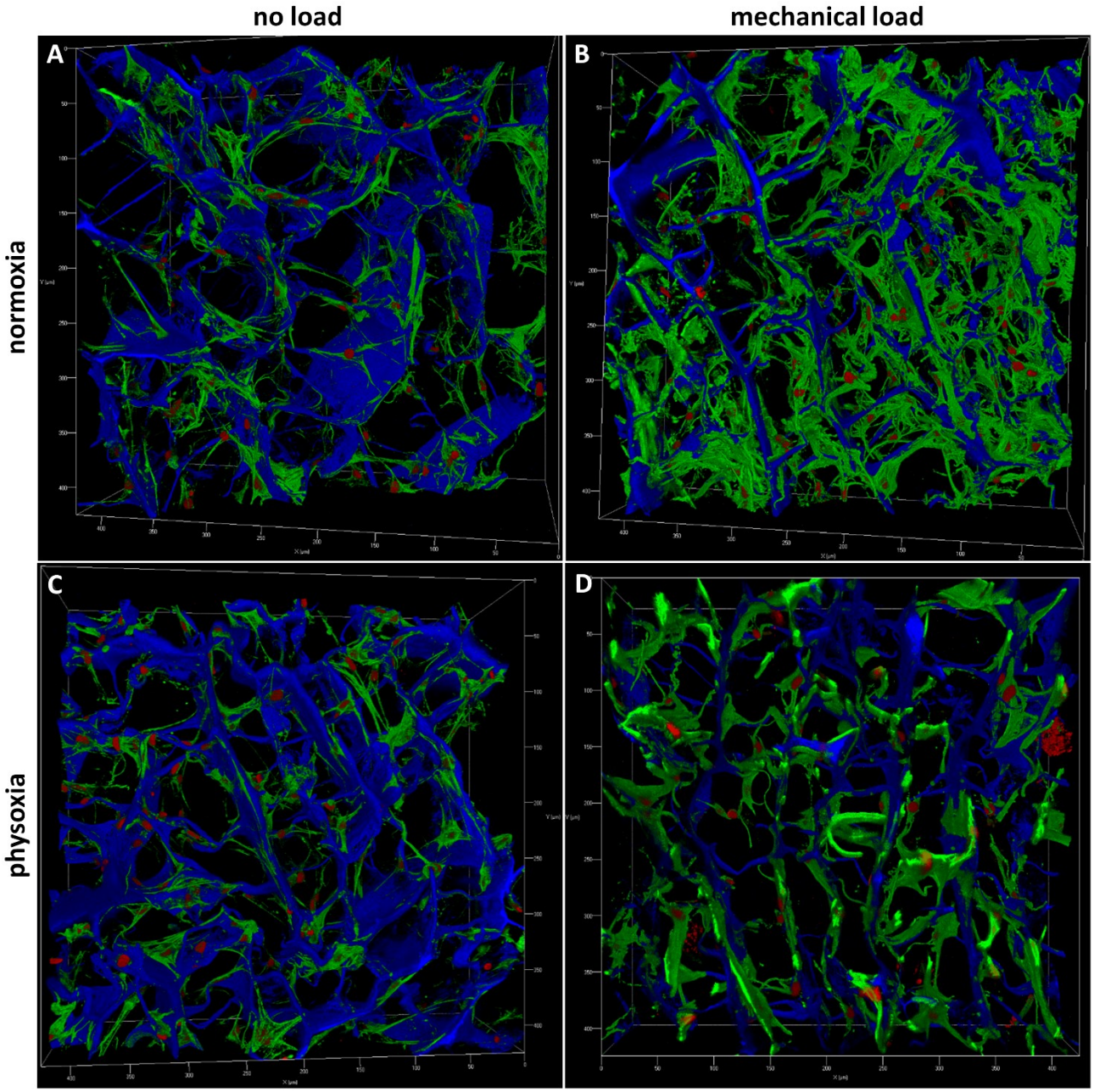



**Fig. S7: Second harmonic generation (SHG) and fluorescence imaging of collagen scaffold grown with primary human OBs in the MPS for seven days.**

Collagen scaffolds were detected by label-free SHG imaging (blue), while cell nuclei and the actin cytoskeleton were stained with SYTOX orange (red) and phalloidin-iFluor 647 (green), respectively. Z-stacks were acquired at the scaffold surface with a 20× dipping objective at 870 nm intervals, comprising (A) 223 µm, (B) 147 µm, (C) 153 , (D) 111 µm in depth. 3D surface rendering was performed. The four samples were prepared with cells from donor 3. Each stack is supplied as supplementary video (S1 – S4). Image dimensions (x, y) 425 x 425 µm.

***Fig. S8:*** *qPCR-based analyses of gene expression at mRNA level*

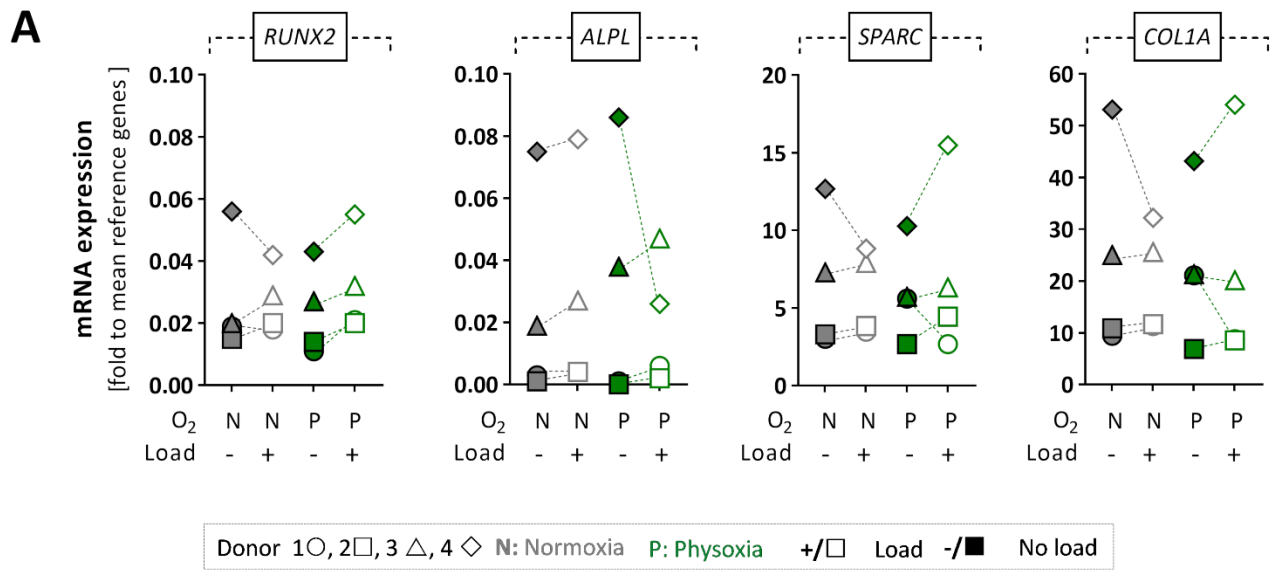
*Method - Analysis of gene expression profiles using real-time quantitative PCR (qPCR)*

To analyse gene expression under different conditions, total RNA was isolated from half scaffolds after seven days of cultivation. Specimens were placed in screw-capped tubes with ceramic beads and snap-frozen in liquid nitrogen. One mL of peqGOLD TriFAST was added, and scaffolds were homogenised in a Minilys bead mill (3x30 s). Lysates were kept frozen until RNA isolation according to the manufacturer's instructions (TRIzol, Thermo Fisher Scientific, Waltham, MA, US). RNA concentrations were determined measuring UV light absorption at 260 nm with a micro-volume UV-Vis spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific, Waltham, MA, US). cDNA was synthesised from total RNA by reverse transcription polymerase chain reaction (RT-PCR) using the High-Capacity cDNA Reverse Transcription Kit according to the manufacturer's instructions.

Finally, to investigate mRNA expression levels of selected marker genes, a panel of nine primer pairs (Fig. S4C) was chosen, and qPCR was performed in 384-well plates using a QuantStudio 7 Flex Real-Time-PCR System (Thermo Fisher Scientific, Waltham, MA, US) in technical triplicates. Gene expression data were analysed with QuantStudio 7, a software for qPCR analysis (Thermo Fisher Scientific, Waltham MA, US). The relative gene expression levels were calculated employing the delta cycle threshold (ΔCT) method. Two reference genes, HSC70 and B2M, were chosen as internal reference for normalisation. The ΔCT value of the gene of interest was calculated by subtracting the mean CT value of the reference genes from the CT value of the gene of interest. The $2^{-\Delta CT}$ value describes the fold change of the mRNA expression levels relative to the mean of the reference genes.

*Results*

Expression of various genes involved in osteogenesis was examined and plotted as a fold change compared to the mean value of the reference genes β2-microglobulin (B2M) and heat shock protein 70 (HSC70). Similar to other datasets in this study, mRNA expression level show a considerable variability between the different donors. The conclusions that can be drawn from this data are therefore limited. In particular, mechanical load seemed to positively influence the expression of runt-related transcription factor 2 (RUNX2), tissue non-specific alkaline phosphatase (ALPL), osteonectin (SPARC) and alpha-1 type I collagen (COL1A1) in some of the donors. SPARC and COL1A1 were expressed at lower levels under physoxia as compared to normoxic conditions, while no clear trend was evident for RUNX2 and ALPL. In summary, we could show that in the MPS osteogenic genes were mostly upregulated under mechanical load in comparison to the corresponding conditions without load.




**B**

| Gene name | Direction | Sequence |
|---|---|---|
| **RUNX2** | forward | AGT CAG ATT ACA GAC CCC AGG |
| | reverse | TTA CTG AGA GTG GAA GGC CA |
| **ALPL** | forward | CCA AGT ACT GGC GAG ACC AA |
| | reverse | TGT GGA GAC ACC CAT CCC AT |
| **SPARC** | forward | GAG GTA TCT GTG GGA GCT AA |
| | reverse | GAA GAG TCG AAG GTC TTG TT |
| **COL1A** | forward | GAA TGG AGA TGA TGG GGA AG |
| | reverse | TCA TTT CCA CGA GCA CCA |
| **B2M** | forward | CTC CGT GGC CTT AGC TGT G |
| | reverse | TTT GGA GTA CGC TGG ATA GCC |
| **HSC70** | forward | TTC TTT GCG GCA TCA CCG ATC AAC |
| | reverse | TCC AAG GGA CCT GCA GTT GGT ATT |

**Fig. S8: qPCR-based analyses of gene expression at mRNA level**
mRNA expression of specific osteogenic markers shown as fold change to the mean of the reference genes B2M and HSC70. All parameters were measured for the same four donors 1○, 2□, 3△, 4◇, either under normoxia (N) or physoxia (P), with (+/□) or without (-/■) mechanical load. **(B)** Primer sequences used in qPCR analyses. Confidence intervals and p-values obtained by nonlinear mixed model analyses are given in Tab. S4A - D. P values < 0.005 were considered as statistically significant. Different significance levels are indicated as: # p<0.05; * p<0.005; ** p<0.0005; *** p<0.0001.

**Tab. S4: Confidence intervals and p-values of the statistically analysed data sets of osteogenic marker genes**

Confidence intervals and p-values of statistical analyses of the datasets obtained from measurements of gene expression. All analysed raw data were from n=4 donors. All parameters were measured under either normoxia or physoxia, and in the presence (load) or absence of mechanical loading. The calculation of confidence intervals and p-values is described in the Experimental. Blue shading indicates effects with a significance level of $p < 0.005$.

**A**

| Effect | Comparison | P-value | △ Confidence interval | | |
|---|---|---|---|---|---|
| | | | LOWER | MEAN | UPPER |
| RUNX2 | normoxia vs. physoxia | 0.6370 | -0.41 | 0.11 | 0.63 |
| RUNX2 | normoxia vs. normoxia load | 0.4610 | -0.70 | -0.18 | 0.34 |
| RUNX2 | physoxia vs. physoxia load | 0.3310 | -0.76 | -0.24 | 0.28 |
| RUNX2 | physoxia load vs. Normoxia load | 0.8230 | -0.47 | 0.05 | 0.57 |
| RUNX2 | physoxia vs. normoxia load | 0.2400 | -0.23 | 0.29 | 0.81 |
| RUNX2 | normoxia vs. physoxia load | 0.6030 | -0.64 | -0.12 | 0.40 |
| | | | Confidence interval | | |
| | | | LOWER | MEAN | UPPER |
| RUNX2 | normoxia | | 4.53 | 5.34 | 6.16 |
| RUNX2 | normoxia load | | 4.35 | 5.17 | 5.98 |
| RUNX2 | physoxia | | 4.64 | 5.46 | 6.27 |
| RUNX2 | physoxia load | | 4.40 | 5.22 | 6.03 |

**B**

| Effect | Comparison | P-value | △ Confidence interval | | |
|---|---|---|---|---|---|
| | | | LOWER | MEAN | UPPER |
| ALPL | normoxia vs. physoxia | 0.3830 | -0.96 | 0.66 | 2.28 |
| ALPL | normoxia vs. normoxia load | 0.9370 | -1.68 | -0.06 | 1.56 |
| ALPL | physoxia vs. physoxia load | 0.1270 | -2.83 | -1.20 | 0.42 |
| ALPL | physoxia load vs. normoxia load | 0.5140 | -2.11 | -0.49 | 1.13 |
| ALPL | physoxia vs. normoxia load | 0.3440 | -0.91 | 0.72 | 2.34 |
| ALPL | normoxia vs. physoxia load | 0.4660 | -2.17 | -0.55 | 1.08 |
| | | | Confidence interval | | |
| | | | LOWER | MEAN | UPPER |
| ALPL | normoxia | | 3.86 | 6.67 | 9.47 |
| ALPL | normoxia load | | 3.80 | 6.61 | 9.42 |
| ALPL | physoxia | | 4.52 | 7.32 | 10.13 |
| ALPL | physoxia load | | 3.31 | 6.12 | 8.93 |

**C**

| Effect | Comparison | P-value | △ Confidence interval | | |
|---|---|---|---|---|---|
| | | | LOWER | MEAN | UPPER |
| COL1A1 | normoxia vs. physoxia | 0.9550 | -0.68 | -0.02 | 0.65 |
| COL1A1 | normoxia vs. normoxia load | 0.9080 | -0.70 | -0.03 | 0.63 |
| COL1A1 | physoxia vs. physoxia load | 0.1170 | -0.16 | 0.51 | 1.17 |
| COL1A1 | physoxia load vs. normoxia load | 0.1060 | -0.14 | 0.53 | 1.19 |
| COL1A1 | physoxia vs. normoxia load | 0.9520 | -0.65 | 0.02 | 0.68 |
| COL1A1 | normoxia vs. physoxia load | 0.1280 | -0.17 | 0.49 | 1.16 |
| | | | Confidence interval | | |
| | | | LOWER | MEAN | UPPER |
| COL1A1 | normoxia | | -5.47 | -4.35 | -3.23 |
| COL1A1 | normoxia load | | -5.50 | -4.38 | -3.27 |
| COL1A1 | physoxia | | -5.48 | -4.37 | -3.25 |
| COL1A1 | physoxia load | | -4.97 | -3.86 | -2.74 |

**D**

| Effect | Comparison | P-value | △ Confidence interval | | |
|---|---|---|---|---|---|
| | | | LOWER | MEAN | UPPER |
| SPARC | normoxia vs. physoxia | 0.9300 | -0.66 | -0.03 | 0.61 |
| SPARC | normoxia vs. normoxia load | 0.5970 | -0.78 | -0.15 | 0.48 |
| SPARC | physoxia vs. physoxia load | 0.4030 | -0.39 | 0.24 | 0.87 |
| SPARC | physoxia load vs. normoxia load | 0.2150 | -0.26 | 0.37 | 1.00 |
| SPARC | physoxia vs. normoxia load | 0.6580 | -0.50 | 0.13 | 0.76 |
| SPARC | normoxia vs. physoxia load | 0.4510 | -0.41 | 0.22 | 0.85 |

| | | Confidence interval | | |
|---|---|---|---|---|
| | | LOWER | MEAN | UPPER |
| SPARC | normoxia | -3.46 | -2.53 | -1.60 |
| SPARC | normoxia load | -3.62 | -2.68 | -1.75 |
| SPARC | physoxia | -3.49 | -2.56 | -1.62 |
| SPARC | physoxia load | -3.46 | -2.53 | -1.60 |

## References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