Supplementary information

ARTICLE TITLE: Coupling liver normothermic machine perfusion with mesenchymal stromal cell bioreactors: a proof of concept study in small and large animal models

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1. Supplementary methods

1.1. Isolation of adipose tissue (AT)-mesenchymal stromal cells (MSCs) and cell culturing before MSC-bioreactor preparation

Subcutaneous adipose tissue (SAT) of the abdominal region was collected during abdominoplasty (Padua Ethical Committee for Clinical Research approval number: 2892P, 10/06/2013). SAT was minced and digested in collagenase type II solution (1 mg/ml) (Merck KGaA, Darmstadt, Germany) at 37 °C in a water bath with medium shaking for 1 hour. The digested AT was mixed by pipetting, serially centrifuged at 350g for 10 min and filtered through 100 μm cell strainer (Falcon, Corning, NY, USA) to remove collagen fibers and debris. Red blood cells were removed by 5 min incubation of cellular pellet with standard lysis buffer. Cells of the stromal vascular fraction were counted in a Neubauer chamber upon Trypan Blue (0.4%) staining, seeded in DMEM F12 10% FBS (Gibco, Thermo Fisher Scientific, Waltham, MA USA) and grown at 37 °C and 5% CO2. Adipose tisse (AT)-derived MSC) were obtained by subculturing.

To produce the adequate cell amount to be seeded into the bioreactor, AT-MSCs were expanded in 75cm^2 tissue culture flasks (Sarstedt AG & Co. KG, Numbrecht, Germany), at a concentration of 14.3*10^4 cells/ml in 14 mL of αMEM medium (Gibco, Thermo Fisher Scientific) added with 5% Human platelet lysate (SCM142, Merck KGaA), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 μg/ml Amphotericin B (Thermo Fisher Scientific). Cells were expanded for 14 days, with medium substitution every 2 days. At 80% confluence, cells were enzymatically harvested and counted. Cells within passages 2 to 8 (P2 to P8) were exclusively utilized to minimize variability.

1.2. Bioreactor description

The hollow fiber bioreactors (Aferetica srl, Bologna, Italy; Supplementary Figure 1) used in the present research show a cylindrical structure containing bundles of polysulfone fibers to create a surface area suitable for cell growth. There are two distinct compartments: the intracapillary space, which is inside the hollow fibers, and the extracapillary space, which surrounds the fibers within the chamber. The fibers provide a total culturing surface of 500 cm^2 and are composed of membranes with pores measuring 150 nm in diameter. Media flows through the fibers (intracapillary space), while cells are typically cultured in the extracapillary space. This setup allows a selective exchange of molecules and factors between the compartments.

1.3. Liverless perfusion using the NMP platform for small animal models

Liverless perfusion experiments were performed using a customized NMP circuit. It consists of a heated glass reservoir (Harvard Apparatus, Holliston, MA, USA), PVC tubing (BD Switzerland Sarl,

Eysins, Switzerland), a peristaltic pump (Ismatec, Wertheim, Germany), a single-use membrane oxygenator (Micro-1, Kewei Rising Medical, Shenzhen, China) and a heated bubble trap (Harvard apparatus). The perfusion fluid was prepared using the following reagents: Williams Medium E (Gibco, Thermo Fisher Scientific), human albumin (Grifols S.A. Barcelona, Spain), L-glutamine (Gibco, Thermo Fisher Scientific), Antibiotic-Antimycotic (Streptomycin, Amphotericin B, Penicillin, Gibco), insulin (Humalog, Eli Lilly, Utrecht, Netherlands), N-acetylcysteine (Zambon Italia, Bresso, Italy), and sodium taurocholate (Organox, Oxford, UK).

Then, 46.2 ml of Oxyglobin® (HBO2 Therapeutics, Souderton, PA, USA), a veterinary-licensed bovine-derived haemoglobin, was added to the solution to reach a final volume of 120 ml. Once the MSC-bioreactors were connected to the circuit, the perfusion fluid was pumped inside the fibers, with 20 ml/min flow rate. To enhance exchange between the 2 compartments of the bioreactor, continuous countercurrent flows of 5 ml/min were adopted outside the fibers.

1.4. Analysis performed on bioreactor supernatants and perfusate samples collected during liverless NMP

Perfusates were used to assess the concentration of the following molecules of human origin: Indoleamine 2,3-Dioxygenase (IDO), caspase-cleaved keratin 18 (CK18), Galectin-9, Interleukin (IL)-8, IL-33, IL-18, IL-1ra, and nitric oxide (NO).

1.4.1. Perfusate sample processing

Perfusate samples were centrifugated at 733 g for 10 min at 4°C (Heraeus Multifuge X3R, Thermo Fisher Scientific). Supernatants were collected and purified with Amicon Ultra 100K centrifugal filter devices (Millipore, Merck KGaA) to avoid colorimetric interferences possibly given by Oxyglobin®. Briefly, 500 µl of each sample were loaded onto the columns and centrifuged at 19357 rpgm for 15 min (Heraeus Multifuge X3R). Next, eluates were collected and stored at -80°C for subsequent biomolecular analysis.

1.4.2. Human Indoleamine 2,3-Dioxygenase (IDO)

The amount of Indoleamine 2,3-Dioxygenase (IDO), was investigated in perfusates and supernatants using a Human Elisa Kit (Invitrogen, Thermo Fisher Scientific). Standards were prepared with a serial dilution ranging from 200 ng/ml to 0.819 ng/ml. Absorbance readings at 450 nm was performed using a multi-mode microplate reader (Synergy HTX, Biotek U.S, Winooski, VT, USA).

1.4.3. Human caspase-cleaved keratin 18 (CK18)

The M30 Elisa kit (VLVbio AB, Nacka, Stockholm, Sweden) is animmunoassay for quantitative determination of the caspase-cleaved keratin 18. The M30 antibody recognizes a neo-epitope exposed after caspase cleavage of K18 fragments containing the K18Asp396 neo-epitope. Standard curve

range was from 1.56 mU/ml to 100 mU/ml. Absorbance readings at 450 nm was performed using a multi-mode microplate reader (Synergy HTX).

1.4.4. Total nitric oxide (NO) metabolites

Concentration of nitric oxide (NO) was evaluated through assessment of total nitrites and nitrates using Griess reagent. A colorimetric commercially available kit with a detection range of 10–100 μM was utilized (Sigma-Aldrich, Merck KGaA). Samples were loaded into Amicon Ultra 10 K centrifugal filter devices (Millipore, Merck KGaA) and then subjected to 15-min centrifugation at 14.000 *g* (Heraeus Multifuge X3R). Next, protein- and hemoglobin-free eluates were added to a nitrate reductase according to manufacturer's instructions. Absorbance readings at 450 nm was performed using a multi-mode microplate reader (Synergy HTX).

1.4.5. Assessment of 8-hydroxy-2'-deoxyguanosine (8-OHdG)

Generation of the oxidized form of 2'-deoxyguanosine was determined to assess the occurrence of oxidative damage to DNA (DNA damage competitive ELISA, Thermo Fisher Scientific). Absorbance reading was performed at 450 nm using a multi-mode microplate reader (Synergy HTX).

1.5. Cell detachement from the bioreactor and flow-citometry

At the end of NMP, MSC-bioreactors were washed with 500 ml of PBS 1x. Then, the devices were disconnected from the circuit and were subjected to enzymatic digestion using 0.25% Trypsin-EDTA (Thermo Fisher Scientific) at 37°C, with vortex mixing at 17 g every 10 min. After 30 min-incubation, supernatants were collected and centrifuged at 413 g for 10 min at 4°C 2 ml PBS 1x. Cell pellets were suspended in 2 ml PBS 1x and counted using the Scepter™ cell counter (Merck KGaA) equipped with 60 μm sensors. Average yield was 10^6 cells. Next, MSCs were cultured in vitro until 90% confluence. Culturing conditions were similar to those adopted for MSC expansion (14.3 x10^4 cells/ml in 75cm^2 flasks (Sarstedt), in 14 ml of complete medium. After an average of 9±3 days, cells were enzymatically detached, centrifuged, washed with ice-cold FACS buffer (2% BSA in PBS 1x), and then an aliquot containing 1.5 x10^6 cells was incubated in the dark for 10 min at room temperature with the following monoclonal mouse anti-human fluorochrome-conjugated antibodies: CD29 Purified (BD Biosciences, Cat No. 556048), CD73-PE (BD Biosciences, Cat No. 550257), CD90-APC (BD Biosciences, Cat No. 559869), CD105-FITC (Fitzgerald, Acton, MA, Cat No. 61R-CD105-DHUFT), CD45-APC-Cy7 (BD Biosciences, Cat No. 348805), CD31-PE-Cy7 (Biolegend, San Diego, CA, Cat No. 303117), CD34 APC-Alexa 700 (Beckman Coulter, Cat No. A86354) Next, cells were washed and incubated with streptavidin- PE-Texas Red (ECD, Beckman Coulter, Cat No. IM3326). Labeled cells were resuspended in 200 μl of FACS buffer and acquired (30000 events/sample) by a FACSCanto™ Flow Cytometer (BD Biosciences). Data acquisition and analysis

were performed using BD FACSDiva™ software. MSCs were identified as CD29+, CD73+, CD90+, CD105+, CD45-, CD31-, CD34-, as reported in the "Guidelines for Standardization and quality assessment for clinical grade MSCs from human adipose tissue" formulated by T. Debnath and coworkers [1].

1.6. Animals and sample size calculation for the rat model

Animals received humane care in compliance with the European Union Directive 2010/63/EU and the Italian Legislative Decree 26/2014. Experiments were planned according to the Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE) guidelines [2] and performed in compliance with the 3R principles [3,4].

An "a priori" power analysis was carried out to estimate the minimum number of rats needed to reliably detect the expected effect size [2–4]. Given the well-known negative effects of reperfusion on mitochondrial oxidative phosphorylation, ATP liver content was used as reference variable to explore the effects exerted by the bioreactor-based perfusion. Details on sample size calculation are listed below:

- number of groups: 3 (Native, NMP, NMP+bioreactor);
- statistical test: one-way or two-way repeated measures ANOVA;
- expected effect size: 0.45;
- expected standard deviation of residuals: 0.20;
- desired power: 0.80;
- alpha error: 0.05.

The analysis provided a minimum number of 5 livers for each experimental group. The final number needed to perform the whole study was therefore 15 rat livers.

1.7. Anesthesia, Surgery, In-situ Perfusion, and rat liver procurement

All experiments were performed in sterile conditions. Rats were anesthetized using Isoflurane (Iso-Vet, Piramal Critical Care, Voorschoten, Netherlands) 3-4% (1-2 L/min O2 flow) in a rat anesthetic induction chamber for 10 min. When unresponsive to pain, rats were placed onto the operating table, ensuring spontaneous breathing with a mask that insufflate 1 L/min O2 and Isoflurane 3-4%.

The surgical procedure began with a xifo-pubic and bilateral subcostal incision. The hepatic pedicle was then exposed, and the bile duct was dissected. Bile duct was distally ligated with an 8-0 nylon tie, then a customized cannula (tip: 24 G Neo Delta Ven, Delta Med SpA, Viadana, Italy; tube: PE-50, BD Intramedic™, Franklin Lakes, NJ, US) was inserted at the end of the choledotomy; the cannula was secured with a proximal 8-0 nylon tie. Then, the pyloric vein was ligated (8-0 nylon) and transected, and then the main trunk of the portal vein was dissected distally. The portal vein was

encircled with three 5-0 silk tie; then unfractioned heparin (2 IU/g diluted in 1 ml of saline from Epsoclar, Pfizer Italia S.r.l., Latina, Italy)) was administered by tail intravenous injection. After three min, portal vein was ligated distally to the splenic vein insertion and a 16 G cannula (Neo Delta Ven, Delta Med SpA) was inserted by venipuncture until the tip of the cannula reached the portal bifurcation. A blood retrograde flush was performed, and the in-situ perfusion system was connected. Then, the cannula was secured with a two-silk tie and gently retracted to avoid obstruction of the right lobe portal vein branch. Rapid sternotomy was performed, the heart was removed, and the inferior vena cava was transected. In-situ cold perfusion (4°C) was started with 35 mL of Celsior solution (IGL, Lissieu, France) at a pressure of 30 cmH2O. At the end of in-situ cold perfusion, hepatectomy was completed and the liver graft was stored in 4°C Celsior (IGL) solution for 30 min. At the end of cold storage, backtable was completed, while maintaining the liver on a refrigerated surface.

1.8. Rat liver normothermic perfusion

At the end of static cold storage (SCS), the liver was placed onto the modified heated glass reservoir to let the liver laid on the diaphragmatic surface on a perforated Parafilm. The liver was then connected to the circuit through the portal vein cannula, while the bile duct cannula was connected to a 1.5 ml tube in order to collect bile during reperfusion. In the advanced protocol, the MSC-bioreactor was attached to the circuit before liver connection. The chamber was closed to maintain humidity. We carefully avoided air embolism during priming and liver connection. The membrane oxygenator was ventilated with 50% FiO2 and 5% $FiCO_2$, 200 ml/min gas flo.

The NMP-protocol was maintained for 280 min and included two phases: 1) the rewarming phase, lasting 40 min and 2) the normothermic phase, lasting 240 min. During the “rewarming” phase, the system reaches the temperature of 37°C. Portal flow was set at 5 ml/min and was increased every 5 min up to 30 ml/min, or until the portal pressure reaches 8 mmHg. In the normothermic phase, temperature was maintained at 37°C and portal flow remained unchanged.

Perfusion fluid volume was maintained at 100 ml throughout the procedure. To prevent MethOxyglobin accumulation, from the beginning of normothermic phase a fixed volume of perfusate was replaced with fresh perfusion fluid at 20 ml/h flow rate. Waste flow rate was 10 ml/h from 0 h to 2 h, while it was increased to 14 ml/h from 2 h to 4 h to adjust for the volume of perfusate collected for biomolecular analysis.

Hemodynamics parameters were monitored using the Powerlab 16/35 system (ADInstruments, Dunedin, New Zeland) and LabChart 8 pro (ADInstruments). Portal vein and pre-membrane oxygenator pressures were recorded every 5 min during rewarming, and every hour during normothermic machine perfusion. Vascular resistances were calculated as mean portal vein pressure

divided by blood flow of the portal vein (ml/min). Temperature was monitored using a thermo probe located between the lobes of the liver. A modified Fick equation was used to calculate oxygen delivery (DO_2) and oxygen consumption ($\dot{V}O_2$) in our experiments. Cardiac output was intended as the pump flow; pre-liver perfusate samples were intended as O_2 enriched perfusate (arterial blood of the Fick equation), whereas post-liver perfusate samples, collected directly from the inferior cava vein, were used for the calculation of venous oxygen content.

$$[1{,}394 \times Hb\ (g/dL)_{PRE\text{-}LIVER} \times HbO_2(\%)] + 0{,}003 \times P_{PRE\text{-}LIVER}O_2(mmHg) =$$

Oxygen content of the pre-liver perfusate ($C_{PRE}O_2$)

$$[1{,}394 \times Hb\ (g/dL)_{POST\text{-}LIVER} \times HbO_2(\%)] + 0{,}003 \times P_{POST\text{-}LIVER}O_2\ (mmHg) =$$

Oxygen content of the post-liver perfusate ($C_{POST}O_2$)

Oxygen delivery was measured as follows:

$$\mathbf{DO_2} = C_{PRE}O_2 \times \text{Pump flow (ml/min)} / 100$$

Similarly, $\mathbf{\dot{V}O_2} = (C_{PRE}O_2 - C_{POST}O_2) \times \text{Pump flow (ml/min)} / 100$

1.9. Processing of perfusate samples collected during rat liver NMP

Samples were centrifugated at 733 g for 10 min at 4°C (Heraeus Multifuge X3R). Supernatants were collected and stored at −80°C for further analysis, whereas cell pellets were washed using 15 ml of PBS 1X, vortexed, and then centrifuged for 10 min at 4°C. Afterwards, pellets were resuspended in 7-15 ml, depending on the volume of perfusate, of erythrocyte lysis buffer (0.155 M NH4Cl, 10 mM KHCO3, 0.1 mM Na2EDTA, pH 7.4) and incubated for 15 min at 4°C. A 10-min centrifugation at 733 g was then performed (Heraeus Multifuge X3R). Pellets were suspended in 0.5-4 mL of PBS 1X for cell count using an automated cell counter (Scepter™, Millipore, Merck KGaA). A qualitative assessment of individual donor cell populations was then performed using the Scepter™ 2.0 software pro (Merck KGaA) by setting the following gates [5]: 1) debris, 3 - 4 μm; 2) cell fragments, 4.5 – 6.6 μm; 3) lymphocytes, 7.0-8.0 μm; 4) monocytes/neutrophils/endothelial cells/MSCs, 9 -18 μm.

To avoid colorimetric interferences possibly given by Oxyglobin®, perfusates were purified with Amicon Ultra 100K centrifugal filter devices (Millipore, Merck KGaA) at 19357 g for 15 min (Heraeus Multifuge X3R).

1.10. Comparative analysis of perfusates collected during liverless NMP and rat liver NMP

Perfusates collected during liverless NMP and liver NMP were used to assess the concentration of the following molecules of human origin: IDO, IL-4, IL-6, IL-8, IL-10, IL-13, IL-1ra, IL-18, IL-33, IL-36-beta, Galectin-3, Galectin-9, and CCL-2/MCP-1. The release of extracellular vesicles (EVs) was likewise investigated.

1.10.1. Human IL-4, IL-6, IL-10, IL-13, IL-1ra, IL-36-beta, Galectin-3 and CCL-2/MCP-1

The concentration of Human IL-4, IL-6, IL-10, IL-13, IL-1ra, IL-36-beta, Galectin-3 and CCL-2/MCP-1 was measured by means Luminex® xMAP Technology (Luminex 200; Luminex, Austin, TX, USA) with a custom-designed immunoassays (R&D Systems). Briefly, 100 µl from each antibody-bead vial were added to a Mixing Bottle and then brought to a final volume of 1 ml with the kit-specific Assay Buffer. Fifty µl of standards, controls and samples were added to the appropriate wells, then each well was brought to a final volume of 100 µL with 50 µl of mixed beads. The plate was incubated in the dark for 2 h with gentle agitation at RT. Plate was washed 3 times for every new step; then Biotin and Streptavidin were added subsequently to the wells. Lastly, 100 µl of Wash Buffer were added for fluorescence detection.

1.10.2. Human Galectin-9

Concentration of Human Galectin-9 was investigated in perfusate samples before and during NMP with an ELISA kit. (R&D Systems, Bio-Techne, Minneapolis, USA). Standards were prepared with a serial dilution ranging from 10 ng/ml to 0.156 ng/ml. Absorbance was read at 450 nm (Synergy HTX).

1.10.3. Human IL-8, IL-33, and IL-18

Pre-configured Ella® (Bio-Techne) cartridges were used to investigate Human IL-8, IL-33, and IL-18. Samples were adequately pre-treated and then loaded onto an Ella cartridge. All sample specific information were entered into the Simple Plex Runner software. The cartridge was inserted into the device and the run was started. At the end of the assay, the results of triplicate measurements in relative fluorescence units, mean signal values, SD and coefficient of the variance (CV) were automatically displayed for each analyte.

1.10.4. Analysis of extracellular vesicles (EVs) released in the perfusate

EVs were isolated from perfusate samples using qEV/35nm size exclusion chromatography columns (Izon Science, Medford, MA), which preferentially isolates EVs between 35-350nm. Isolated EVs then underwent size and concentration measurements by nanosight tracking analysis using a Nanosight NS300 (Malvern Instruments, UK).

1.11. Analysis of perfusate samples collected during rat liver NMP

After perfusate processing as reported in the paragraph 1.8.1, samples were used to assess the following parameters: aspartate aminotransferase (AST), alanine aminotransferase (ALT), Lactate dehydrogenase, (LDH), NO, rat biomarkers of liver injury, rat biomarkers of endothelial activation, rat inflammation-related mediators, and rat molecules involved in inflammation resolution and liver cell regeneration.

1.11.1. Succinate

Perfusate succinate was assessed using the Succinate Colorimetric Assay Kit (Sigma-Aldrich,). Perfusates were deproteinized using Amicon Ultra 10K centrifugal filter devices (Millipore, Merck KGaA). Standards were prepared directly in wells adding from 10µl to 2µl of the 1mM Standard Solution, therefore with a scale that ranged from 10 nmol/well to 2 nmol/well. After a 30-min incubation at 37°C, absorbance was read at 450 nm (Synergy HTX).

1.11.2. NADH

NADH concentration in perfusates was analyzed trough fluorescence intensity, as described elsewhere [6]. A pre-weighed NADH vial was purchased from Sigma Chemical Company (St. Louis, MO). A stock solution of 10 mg/ml was prepared using Na-bicarbonate buffer, pH 9. A standard curve ranging from 250 to 2.5 ng/ml was then prepared using filtered perfusate/medium. Seventy-five µl of each sample were then added in duplicates to the wells. Fluorescence was determined using a 340 nm excitation filter and a 440 nm emission filter using a multiplate reader (Synergy HTX).

1.11.3. Molecules relevant to tissue damage, inflammation and resolution

Soluble proteins relevant to liver damage, vascular damage and immune activation were evaluated by means of Luminex® xMAP Technology (Luminex 200) with the following custom-designed immunoassays (Millipore, Merck KGaA): 1) Rat Liver Injury Magnetic Bead Panel (Arginase-1: ARG1, 5'-nucleotidase: 5'-NT, Glutathione Transferase Alpha: GST-alpha; Sorbitol dehydrogenase: SDH); 2) Rat Vascular Injury Magnetic Panels (Adiponectin; Caveolin 1:CAV-1, Connective Tissue Growth Factor: CTGF, Tissue Plasminogen Activator Inhibitort-1: t-PAI-1, soluble Intercellular Adhesion Molecule-1: sICAM-1, Soluble endothelial leukocyte adhesion molecule-1: sE-Selectin, Alpha-1-acid glycoprotein: AGP, Alpha-2-Macroglobulin: A2M, Haptoglobin); 3) Rat Cytokine/Chemokine Magnetic Bead Panel (Macrophage Inflammatory Protein-1alpha MIP-1alpha; Interleukin-4: IL-4, Interleukin-6: IL-6, Interleukin-10: IL-10, Interferon-gamma: IFN-gamma, Interleukin-18: IL-18, Chemokine C-C motif ligand 2/Monocyte Chemoattractant Protein-1: CCL2/MCP-1, Interferon gamma-induced protein 10: IP-10, C-X-C motif chemokine ligand 1/Cytokine-Induced Neutrophil Chemoatractant-1: CXCL1/CINC-1, Vascular Endothelial Growth

Factor: VEGF, C-X-C Motif Chemokine Ligand 5, CXCL5/LIX, Tumor Necrosis Factor-alpha: TNF-alpha, Chemokine C-C motif ligand 5/regulated on activation, normal T cell expressed and secreted: CCL5/RANTES).

Briefly, 150 µl from each antibody-bead vial were added to a Mixing Bottle and then brought to a final volume of 3 ml with the kit-specific Assay Buffer. Twenty-five µl of standards, controls and samples were added to the appropriate wells, then each well was brought to a final volume of 75 µl with 25 µl of Assay Buffer and 25µl of Mixed Beads. The plate was incubated in the dark for 2 h with gentle agitation at RT. Plate was washed 3 times, then Detection Antibodies and Streptavidin-Phycoerythrin were added to the wells. Lastly, 150 µl of Sheath Fluid were added for subsequent fluorescence detection using a Luminex 200 (Luminex).

1.11.4. Rat Tissue Inhibitor of Metalloproteinase 1 (TIMP-1)

The amount of the Tissue Inhibitor of Metalloproteinase 1 (TIMP-1), was investigated in perfusate samples using a Rat TIMP-1 Immunoassay (R&D Systems). Standards were prepared with a serial dilution ranging from 2400 pg/ml to 37.5 pg/ml. Absorbance was read at 450 nm with wavelength correction at 540 nm (Synergy HTX).

1.11.5. Rat IL-13, Hepatocyte growth factor (HGF)

The amount of IL-13 was investigated in perfusates using an ELISA kit (Abcam, Cambridge, UK). Standards were prepared with a serial dilution ranging from 5000 pg/ml to 78.125 pg/ml. Absorbance readings at 450 nm was performed using a multi-mode microplate reader (Synergy HTX).

HGF was investigated in perfusates using a Rat HGF Immunoassay (R&D Systems). Standards were prepared with a serial dilution ranging from 4000 pg/ml to 62.5 pg/ml. Absorbance was read at 450 nm with wavelength correction at 540 nm (Synergy HTX).

1.12. Evaluations performed on rat liver biopsies

1.12.1. Wet-to-Dry Ratio

Biopsies were weighted on an analytical balance (Sartorius AG, Göttingen, Germany) before and after desiccation in an oven at 50 °C for 48 h. Thereafter, wet/dry ratios (W/D) were calculated as an index of edema.

1.12.2. ATP content assessment

Snap frozen liver samples were homogenized in trichloroacetic acid (Sigma-Aldrich) at a concentration of 50 mg/ml. Liver homogenates were subjected to centrifugation at 20817 g for 15 min at 4°C (Heraeus Multifuge X3R) and then supernatants were collected and diluted 1:30 using 0.1 M Tris-acetate, pH 7.75 (Millipore, Merck KGaA). Next, 10 µl of each sample were dispensed in a

blank 96-well plate and 90 µL of luciferase (Enliten ATP Assay System, Promega, Madison, WI, USA) were automatically added during the analysis in the Luminometer (Glomax Luminometer, Promega). Bioluminescent signals were immediately detected. ATP concentration was calculated by means of a standard curve that ranged from 10^{-4} M to 10^{-10} M (rATP 10 mM, Promega).

1.12.3. Determination of ATP derivatives by High-performance liquid chromatography

Snap frozen tissue biopsies (40-50 mg) were homogenized in 2.5% trichloroacetic acid (Sigma-Aldrich) at a concentration of 50 mg/ml. Liver homogenates were subjected to centrifugation at 20817 g for 15 min at 4°C (Heraeus Multifuge X3R) and then supernatants were analyzed in duplicates by High-performance liquid chromatography (HPLC) with a mass spectrometer as detector (UltiMate 3000, Thermo Fisher Scientific) using a reversed phase C18 HPLC column (Zorbax RRHD SB-C18, 5micron, 4.6x250 mm, Agilent, Santa Clara, CA, US), with a mobile phase containing 0.1% formic acid in water (fase A) and CH3CN (fase B). The signal was detected by UV absorbance at 254 nm. Energy charge defined as: (ATP + 0.5 x ADP) / (ATP + ADP + AMP) was calculated as previously described [7].

1.12.4. Tissue NAD+/NADH content measurement

NAD+/NADH content was assessed using a Colorimetric Assay Kit (Sigma-Aldrich). Snap frozen liver samples were homogenized at a concentration of 50 mg/ml. Standards ranged from 10 nmol/well to 2 nmol/well. Absorbance was read at 450 nm (Synergy HTX).

1.13. Porcine model of Donation after cardiocirculatory death (DCD) and liver NMP

1.13.1. Sample size

This pilot study was designed to establish a novel perfusion system by combining the Liver Assist® machine to a stem cell bioreactor. Sample size was determined according to the guidance published by NC3R [8], considering the following possible outcomes: 1) success of execution (feasible) or 2) failure of execution (unfeasible). Based on our experience in the set up and implementation of unprecedent perfusion platforms for preclinical models [9–11], we deemed necessary using 3 animals for the NMP+bioreactor group. Therefore, work package 3 included a total of 6 animals.

1.13.2. Preparation of the bioreactor suitable for porcine livers

Human plasmafilters (Aferetica Srl) were used as bioreactors for the pig experiments (Supplementary Figure 10). Briefly, these devices have a 2000 cm^2 surface area and, similar to the bioreactors used in the small animal study, the fibers are constituted from 150 nm pores-membranes. As established in the first two work packages of the research, bioreactors were prepared in the Padua Lab. A total of

327 3.7 x10^7 stem cells were injected into the extracapillary space of the bioreactor at a seeding density
328 of 1.85 x10^4 cells/cm^2. When adjusted for the perfusate volume and flow rate into the bioreactor, the
329 cell concentration resulted similar between the rodent and porcine models.
330 After 12 h-incubation at standard conditions, supernatants were replaced with complete medium and
331 bioreactors were shipped at room temperature to the Department of Veterinary Medical Sciences
332 (Ozzano, Italy).

333 *1.13.3. Anesthesia, surgery, liver procurement*

334 After an initial sedation with an intramuscular injection of 5 mg/kg Tiletamine-Zolazepam (Zoletil®,
335 Virbac, France), anesthesia was induced using 6-8% Sevoflurane (SevoFlo, Zoetis, Belgium) in 1:1
336 oxygen/air via face mask, then maintained with the same halogenated agent (2-3 %) upon orotracheal
337 intubation. Animals were ventilated and monitored using a fully equipped anesthesia delivery unit
338 (Datex Ohmeda ADU S5, GE healthcare, Chicago, IL, USA). After heparin intravenous
339 administration at a dosing of 300 UI/Kg (Eparina Vister, Teva Italia srl, Italy), the left jugular vein
340 was cannulated (High flow double lumen ST 13 Fr, Joline, Hechingen, Germany) to allow the
341 collection of 2000 ml of autologous blood.
342 At 60 min from death declaration, livers were cold flushed using 2000 ml of Ringer ‘s solution
343 (S.A.L.F. spa, Italy) for 20 min. At the end of in-situ cold perfusion, hepatectomy was completed and
344 the bile duct, the portal vein (PV), and the hepatic artery (HA) were isolated and cannulated. The
345 whole back table procedure lasted 10 min for a total of 30 min cold ischemia time.

346 *1.13.4. Protocol for porcine liver NMP*

347 Livers were connected to an oxygenated dual vessel perfusion device (Liver Assist®, XVIVO
348 Perfusion AB, Sweden), with the following target settings: PV pressure 8-10 mmHg; HA pressure
349 60-70 mmHg; temperature at steady state 37°C.
350 The perfusion fluid consisted in 1000 ml of leukocyte-depleted autologous blood, supplemented with
351 (Infuplas freeflex Fresenius), antibiotics (Fluconazole, Kabipac; Cefazolin, Qilu Pharma), parenteral
352 nutrition (Olimel N4, Baxter), Multivitaminics (Cernevit, Baxter), Heparin, Calcium gluconate
353 (Monico spa, Venezia Mestre, Italy), Sodium chlorate (Monico), Sodium bicarbonate (Monico), and
354 Fast acting Insulin (Humulin-R Lilly). The perfusate final volume was 2000 ml; no fluid replacement
355 was performed over the NMP procedure.
356 According to our clincal protocol, livers were connected to the device through the HA and PV
357 cannulae at 21°C. During the rewarming phase, fluid temperature and flows were gradually increased
358 to reach the pre-set target values over 15 min. The normothermic phase was maintained for 4 h.
359 Following the manufacturer’s instructions, the HA was perfused with a 60 bpm pulse generated by a
360 centrifugal pump. Perfusate oxygenation and/or gas exchange occurred via 2 separate oxygenators.

Hemodynamics parameters and temperature were continuously monitored over the procedure. Gas analysis and biochemical tests of the perfusion fluid were performed at pre-defined time intervals.

2. Supplementary results

2.1. Preliminary characterization of MSCs isolated from adipose tissue

Flow cytometry analysis were performed to characterize the immunophenotype of MSCs isolated from adipose tissue and then cultured in vitro (from P1 to P8).

The analysis revealed a consistent expression of MSC-specific markers as CD90, CD73, CD105 and CD29 on the membrane of the majority of MSCs (91-99%) independently on the in vitro subculturing passage and on the cell cycle staging. On the contrary MSCs were negative for hematological lineage-specific marker (CD45) and endothelial lineage-specific marker (CD31) and downregulated by in vitro culturing the expression of CD34 progressively starting from P2.

2.2. Transmembrane pressure during liverless NMP

Filter pressure drop (ΔP filter) remained low during the perfusion and transmembrane pressure (TMP) was stable, suggesting no clotting or clogging issues over 4 h (Supplementary Figure 1).

2.3. Gas analysis during liverless NMP

Although pH values increased over the liverless procedure, they remained slightly under the physiological range (Supplementary Figure 2). A rise of K+ and Cl- concentration was likewise revealed. Finally, Na+ and Ca++ did not change during the liverless NMP.

2.4. EVs suspended in the perfusates during rat liver NMP

The EVs suspended in the perfusates of the NMP+bioreactor group ranged from 123.80 nm to 209.2 nm, while a different particle size was revealed in the perfusate samples collected during standard NMP (Supplementary Figure 4).

2.5. Release of Human CK18 during liverless and rat liver NMP

We investigated whether MSCs underwent cell death during rewarming, by assessing the release of human CCK18 into the perfusate. The data showed that the degree of apoptosis activation during liver-NMP was similar to that observed during liverless-NMP, indicating that the cells did not experience excessive distress during the different phases of the liver-NMP procedure (Supplementary Figure 5).

2.6. Hemodynamics during rat liver NMP

Hemodynamics monitoring indicated that portal pressures and vascular resistances increased over the NMP procedure ($p<0.001$), but remained below 8 mmHg without adjustments of flow rate.

No differences in both portal pressures and vascular resistances were detected across experimental groups ($p=0.713$ and $p=0.624$, respectively; Supplementary Figure 5).

2.7. Gas analysis of perfusate samples collected during rat liver NMP

A trend towards perfusate pH stabilization was observed in the bioreactor-based perfusion starting from 2 h post-reperfusion, while a progressive pH decrease was observed in the NMP group (NMP+bioreactor: from 6.941±0.02 to 7.048±0.017; NMP: from 6.973±0.021 to 6.992±0.021, p=0.045 at 4 h).

Lactate uptake was calculated with the following formula: $(C_{start} - C_{end}) / C_{end}$ where C stands for concentration. Total lactate uptake was similar between groups (NMP+bioreactor: 1.36±0.25 vs 1.2±0.30, p=0.837)

The results of electrolyte assessment are shown in Supplementary Figure 4. A higher amount of K+ was revealed in the standard NMP procedure compared to NMP+bioreactor procedure. On the other hand, the concentration of Ca++ and Cl- was higher in the NMP+bioreactor group, even if the concentration of both electrolytes slightly varies throughout reperfusion. Na+ concentration is similar between groups.

2.8. Analysis of the bile produced during rat liver NMP

The following bile parameters were similar between the NMP+bioreactor and the NMP group: △pH (NMP+bioreactor vs NMP: 0.028±0.040 vs 0.082±0.020; p=0.340), △HCO3- (3.30±1.71 mmol/L vs 4.0±0.64 mmol/L; p=0.730), and △glucose (49.8±13.99 mg/dl vs 26.4±20.17 mg/dl; p=0.350).

2.9. NAD+/NADH evaluation

The NMP+bioreactor group registered a higher amount of both NAD total and NADH content in tissue homogenates with respect to both native livers and to the NMP group (Supplementary Figure 5). In general, perfusion procedures lead to a higher concentration of NAD+/NADH (One way ANOVA, Tukey's post hoc test. p-value vs the Native group: ***<0.001; p value vs the NMP group: #<0.05; ###<0.001).

The NADH content released in perfusate by livers subjected to the bioreactor-based perfusion was higher compared to that of the NMP group and remained stable throughout reperfusion (Two-way RM ANOVA, Tukey's post hoc test. p-value vs the NMP group: ***<0.001).

2.10. Characterization of the MSCs harvested from the bioreactors after liver NMP

MSCs recovered from the bioreactors at the end of the perfusion experiments displayed similar viability and morphology compared with the original cells. Flow cytometry showed similar MSCs' immunophenotype before and after NMP experiments.

3. Supplementary Tables

Supplementary Table 1. Experimental design adopted to perform rat liver NMP

	Procedure				
Experimental group (n=5)	**Anesthesia**	**Liver procurement**	**In situ perfusion**	**SCS (30min)**	**NMP (240min)**
Native	√	√			
SCS	√	√	√	√	
NMP	√	√	√	√	√
NMP+bioreactor	√	√	√	√	√

Supplementary Table 2. Gas analysis of MSC-bioreactor supernatants

Samples were collected immediately after MSC-bioreactor delivery to the the Center for Preclinical Research, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy (Milan lab). Ten bioreactors were analyzed; Data are presented as mean±SEM.

Parameter	Reference value	MSC-bioreactor supernatants (n=10)
pH	7.49	7.03±0.09
K+, mmol/L	4.8	4.05±0.3
Na+, mmol/L	135	137.54±10.51
Ca++, mmol/L	0.99	0.93±0.01
Cl-, mmol/L	121	125.54±1.23
Lactate, mmol/L	0.7	6.65±0.36
Glucose, mg/dl	101	25.72±3.25

Supplementary Table 3. Timings of sample collection during rat liver NMP

		Wash-out	Rewarming phase	Normothermic phase			
Sample	**Evaluation**	**5 min**	**40 min**	**1 h**	**2 h**	**3 h**	**4 h**
Perfusate							
0.5 ml pre-liver	Gas analysis		√	√	√	√	√
0.5 ml post-liver	Gas analysis	√	√	√	√	√	√
4 ml pre-liver	Bio-molecular analysis	√	√	√	√	√	√
4 ml post-liver	Bio-molecular analysis	√	√	√	√	√	√
3 ml post-liver	Biochemical tests	√	√	√			√
Bile	Gas analysis		√	√	√	√	√
Tissue biopsy	Bio-molecular analysis						√

Supplementary Table 4. Biochemical analysis of perfusate samples collected throughout porcine liver NMP

Variable	Group	Time			p-value
		1 h	2 h	4 h	
Urea, mg/dL					0.657
	NMP	14.33±2.72	56.66±2.02	36.4±2.15	
	NMP+bioreactor	41.66±1.45	13.2±1.77	59.4±5.01	
Creatinine, mg/dL					*0.099*
	NMP	1.26±0.25	1.2±0.15	1.13±0.04	
	NMP+bioreactor	1.32±0.2	1.08±0.04	1.08±0.04	
AST, IU/L					0.102
	NMP	2043±474	3038±1472	1463±184	
	NMP+bioreactor	2670±1304	757±139	1690±236	
ALT, IU/L					0.304
	NMP	80±37.63	53.33±19.41	66.4±11.13	
	NMP+bioreactor	94±45.82	51.4±5.87	56.8±8.81	
Total Bilirubin, mg/dL					**0.039**
	NMP	0.1±0.02	0.18±0.05	0.08±0.02	
	NMP+bioreactor	0.17±0.07	0.06±0.01	0.1±0.03	
GGT, IU/L					0.311
	NMP	10.93±3.12	12.76±3.64	11.4±1.61	
	NMP+bioreactor	15.56±1.98	11.4±0.95	11.3±1.49	
Total Cholesterol, mg/dL					0.199
	NMP	15.33±4.66	14.33±5.23	10±3.34	
	NMP+bioreactor	15±6.42	8.8±3.63	11.6±3.58	
Uric acid, mg/dL					*0.062*
	NMP	0.34±0.2	0.19±0.09	0.08±0.04	
	NMP+bioreactor	0.43±0.25	0.34±0.03	0.01±0	
Glucose, mg/dl					0.885
	NMP	361.3±119.0	588.6±244.2	352.3±123.2	
	NMP+bioreactor	496.4±45.7	477.6±129.3	283.4±88.2	

Three cases were analyzed for each experimental group; Data are presented as mean±SEM. Two-way ANOVA. Abbreviations: ALT, alanine aminostransferase; AST, aspartate aaminotransferase; GGT, gamma glutamyltransferase

Supplementary Table 5. Gas analysis of the perfusate during porcine liver NMP

		Time			
Variable	**Group**	**1 h**	**2 h**	**4 h**	**p-value**
Ca, mg/dL					*0.084*
	NMP	4.2±1.35	4.23±0.71	4.03±0.56	
	NMP+bioreactor	4.7±1.05	6.58±1.3	6.78±1.29	
P, mg/dL					**0.037**
	NMP	7.53±0.61	10.29±0.78	8.81±1.51	
	NMP+bioreactor	6.15±0.05	8.63±0.47	7.81±0.76	
Na, mEq/L					0.414
	NMP	159.33±7.26	158.66±3.92	161.66±1.85	
	NMP+bioreactor	159.4±1.02	155.8±1.31	158.6±1.86	
K, mEq/L					0.209
	NMP	4.83±1.01	2.8±0.09	2.03±0.43	
	NMP+bioreactor	4.56±0.5	4.24±0.34	2.76±0.32	
Cl, mEq/L					**0.008**
	NMP	103.03±5.19	101.43±2.27	103.93±3.65	
	NMP+bioreactor	96.5±0.94	96.9±1.06	98.52±1.23	
Mg, mg/dL					0.187
	NMP	2.29±0.3	1.84±0.59	1.37±0.58	
	NMP+bioreactor	2.39±0.02	2.24±0.1	1.82±0.16	

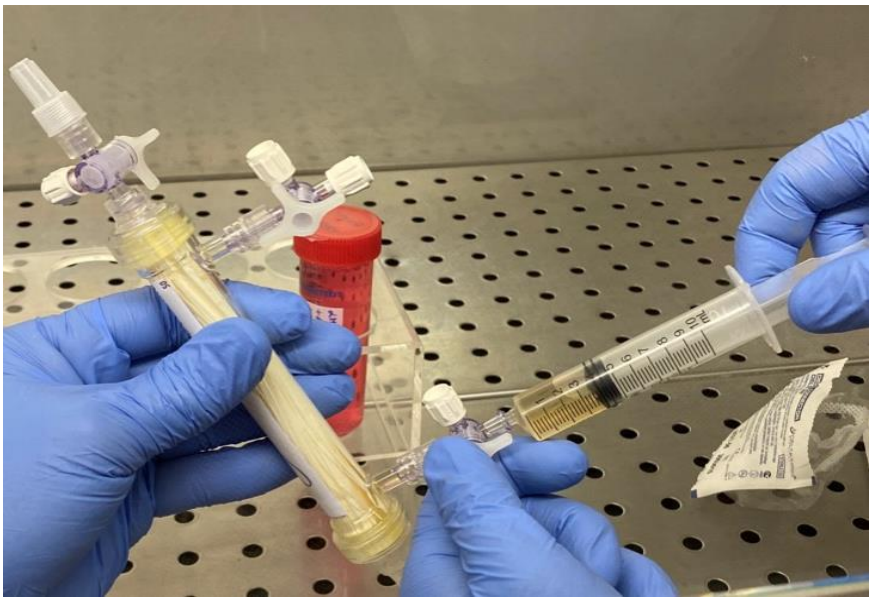
Three cases were analyzed for each experimental group; Data are presented as mean±SEM. Two-way RM ANOVA.

Supplementary Table 6. Histological evaluation of tissue biopsies collected before and after porcine liver NMP

		Parameter				
Group	**Time**	**Hemorrage**	**Necrosis**	**Cholestasis**	**Sinusoidal dilatation**	**Total score**
NMP						
	before	0±0	0±0	0±0	1±0	1±0
	after	0±0	0±0	0±0	1±0	2±1
NMP+bioreactor						
	before	0±0	0±0	0±0	1±0	2±0
	after	1±0	0±0	0±0	1±0	3±1

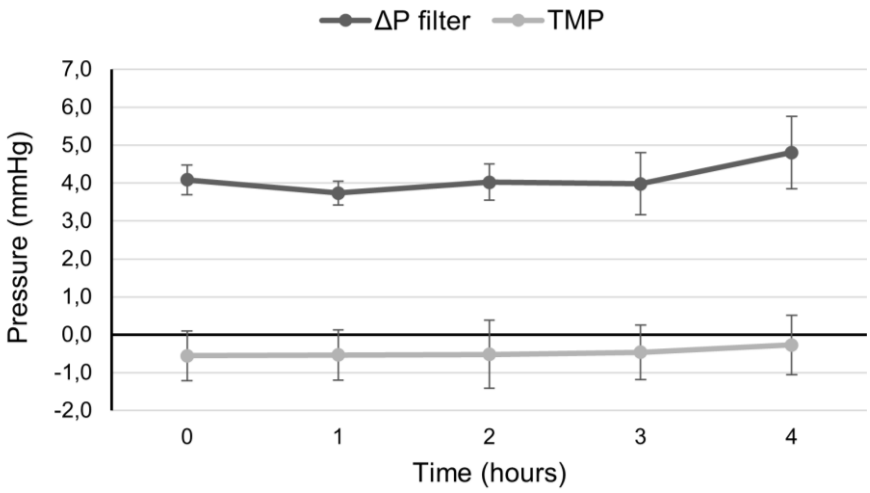
4. Supplementary Figures

Supplementary Figure 1. MSC-bioreactors utilized to develop the advanced NMP platform for small animal models



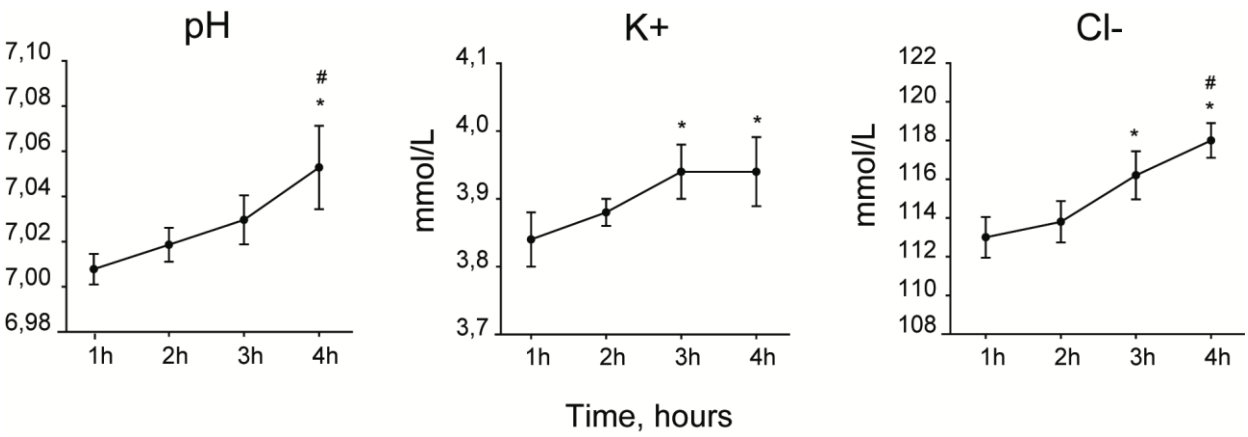
The hollow fiber bioreactors (Aferetica srl, Bologna, Italy) show a cylindric structure with 2 stopcocks per side. There are two compartments: 1) the intracapillary space; 2) the extracapillary space. The fibers provide a total of 500 cm^2-culturing surface and are constituted from 150 nm pores-membranes. The pore size allows a selective exchange of molecules and factors between the two compartments.

Supplementary Figure 2. Transmembrane pressure during liverless NMP



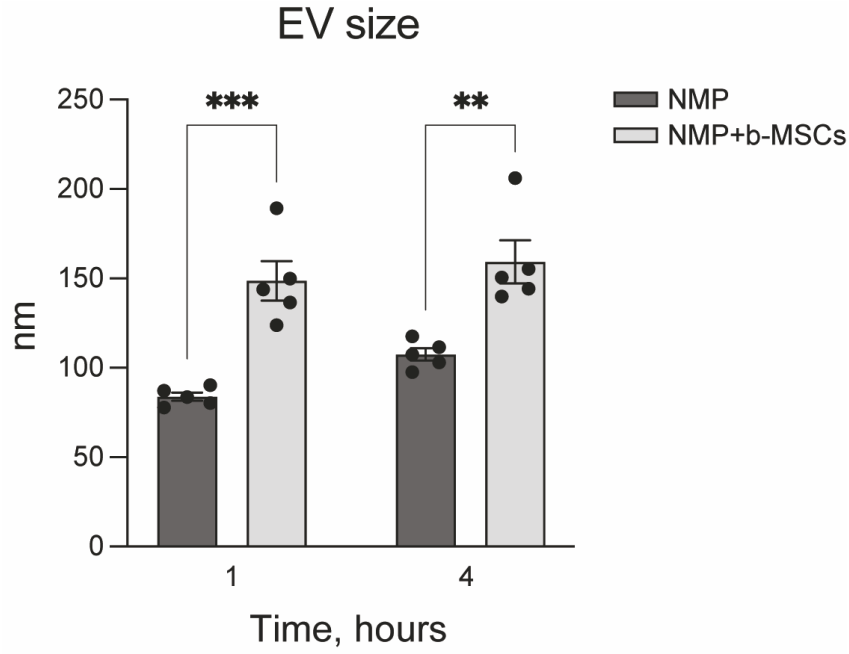
Filter pressure drop (ΔP filter) and Transmembrane Pressure (TMP) were monitored during 4h perfusion to asses preassure parameters stability. Filter pressure drop (ΔP filter) stayed low and Transmembrane Pressure (TMP) remaied stable troughout perfusion. Points denote mean±SEM, n=5 independent liverless procedures.

Supplementary Figure 3. Gas analysis during liverless NMP



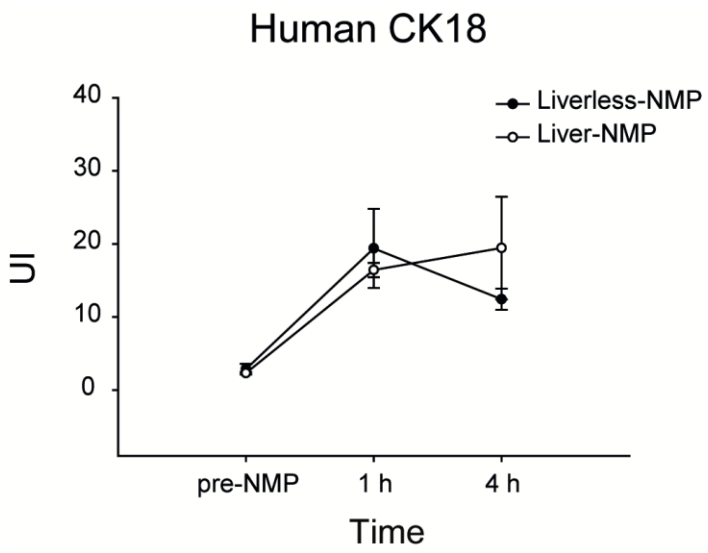
Perfusates gas-analysis during liverless-NMP showed a rise in pH levels, as well as an increased release of K+ and Cl-. Points denote mean±SEM, n=5 independent liverless procedures. One-way RM ANOVA.p-value for time: pH p=0.002; K+ p=0.010; Cl- p=0.001. Asterisks denote: p -value vs 1 h *<0.05; p -value vs vs 2 h #<0.05.

Supplementary Figure 4. Extracellular vesicles realesed during rat liver-NMP



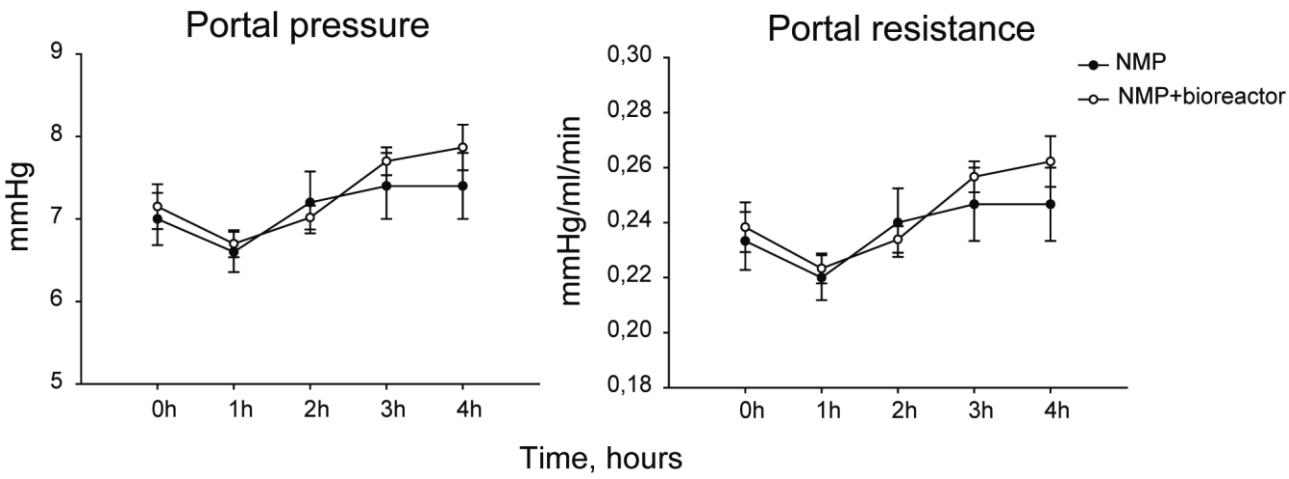
The extracellular vesicles released during the NMP+bioreactor procedure showed a greater size relative to those detected in the perfusates of standard NMP. Bars denote mean±SEM, n=5 independent biological replicates. Two-way ANOVA, Tukey's post hoc test. P-value for group comparison: $p<0.0001$. Asterisks denote p-value vs NMP: ***p=0.003; **p=0.002. Abbreviations: b-MSCs, MSC-bioreactor; EVs, extracellular vesicles; NMP, normothermic machine perfusion.

Supplementary Figure 5. Release of Human CK18 during liverless- and rat liver-NMP



The release of human CK18 was measured as an index of MSCs apoptosis. Pre-NMP samples were collected immediately before MSC-bioreactor connection to the perfusion circuit. No significant differences were detected between experimental groups. Points denote mean±SEM, n=5 independent biological replicates. Two-way RM ANOVA, p=0.613. Abbreviations: CK18, cytokeratin 18; NMP, normothermic machine perfusion.

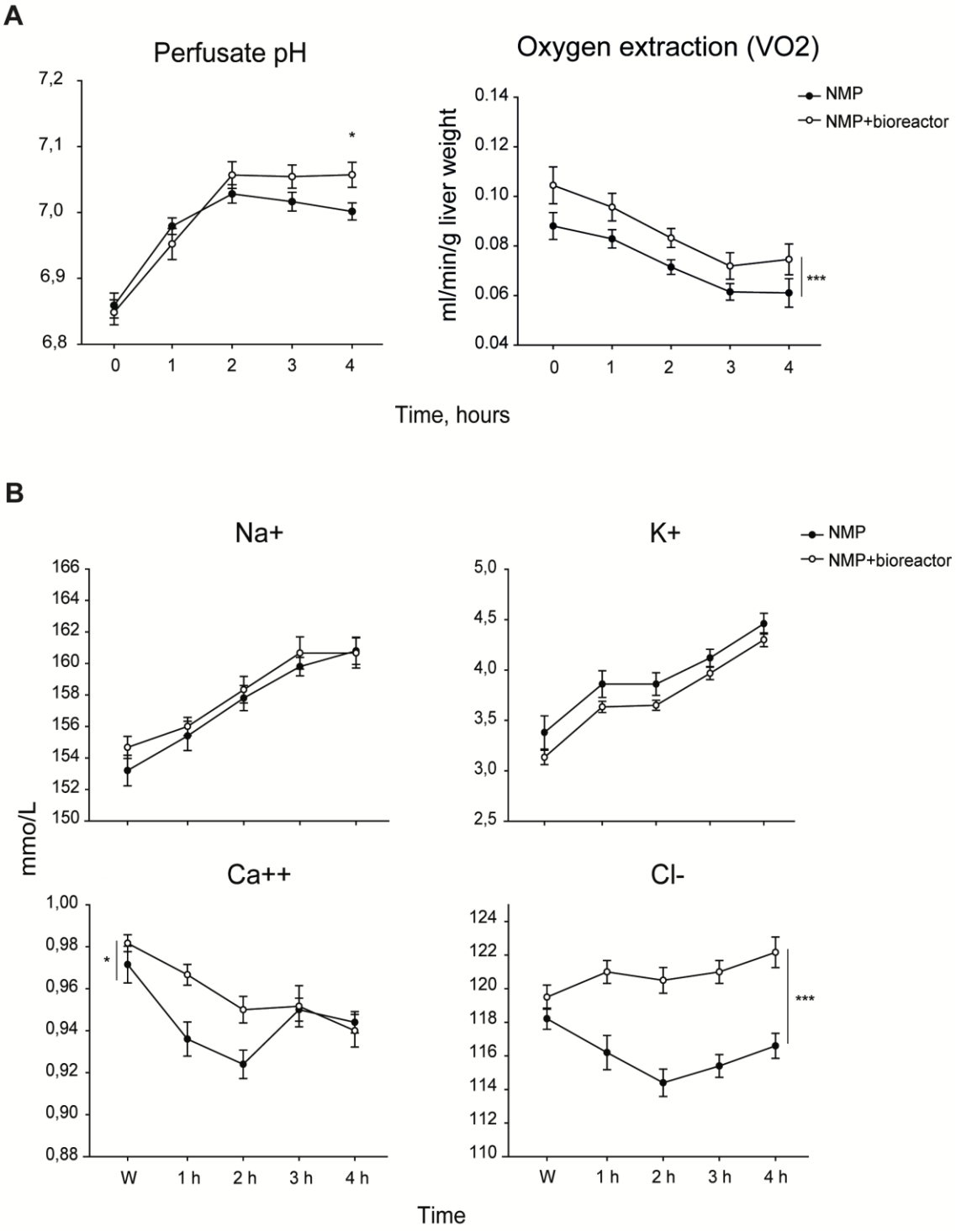
Supplementary Figure 6. Hemodynamics parameters during rat liver NMP



Hemodynamics parameters were monitored throughout liver-NMP. Portal pressures and vascular resistances increased over the procedure, remaining below 8 mmHg.

No statistically significant differences were detected between experimental groups. Bars denote mean±SEM, n=5 independent biological replicates. Two-way RM ANOVA, portal preassure: p=0.713; portal resistance: p=0.624. Abbreviations: NMP, normothermic machine perfusion.

Supplementary Figure 7. Gas analysis of perfusate samples collected during rat liver NMP

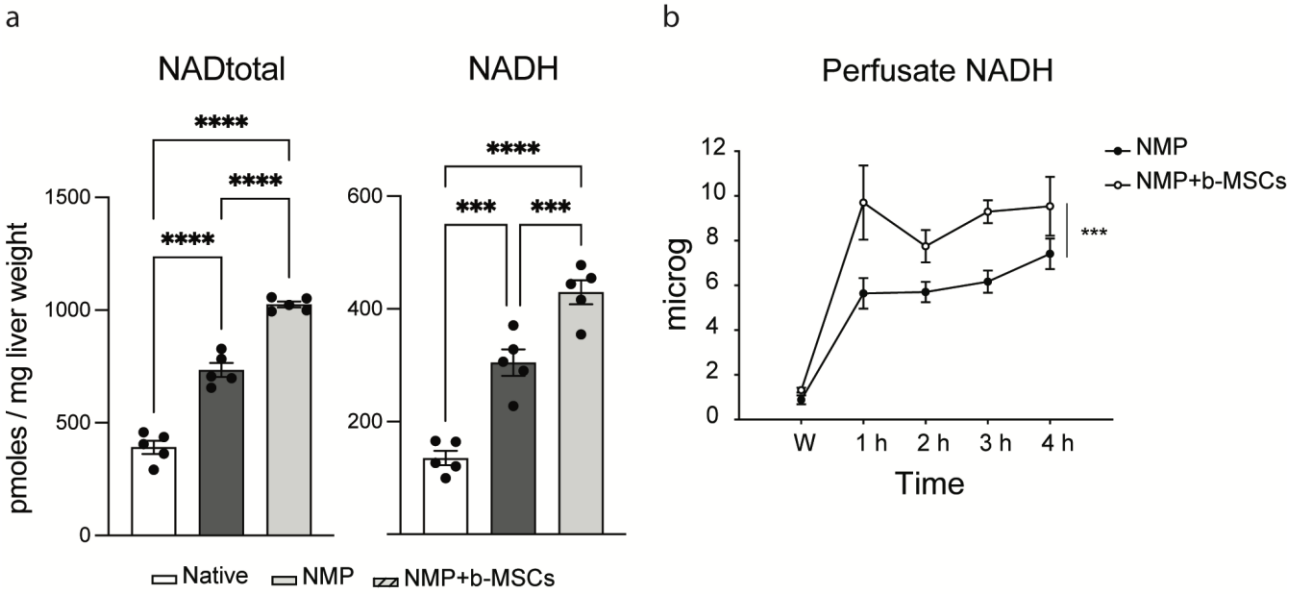


A) Perfusate pH was stable in the NMP+bioreactor group starting from 2 h post-reperfusion, while a progressive pH decrease was observed in the NMP group. Points denote mean±SEM, n=5 independent biological replicates. Two-way RM ANOVA, p-value vs NMP at 4 h: p=0.045. Oxygen extraction (VO_2) was consistently higher in the NMP+bioreactor group compared to NMP alone.

Points denote mean±SEM, n=5 independent biological replicates. Two-way RM ANOVA, p-value vs NMP: ***p<0.001.

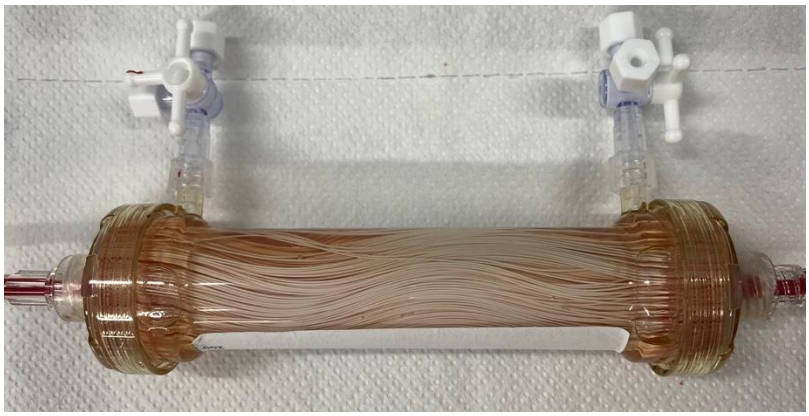
B) Perfusate electrolytes during liver-NMP.. Points denote mean±SEM, n=5 independent biological replicates. Two-way RM ANOVA, p-value for group comparison: K+ p=0.059; Ca++ p=0.027; Cl- p<0.001; Na+ p=0.438. Asterisks denote *p<0.05; ***p<0.001. Abbreviations: NMP, normothermic machine perfusion; W, wash-out.

Supplementary Figure 8. NAD+ and NADH content in rat liver homogenates and perfusate samples



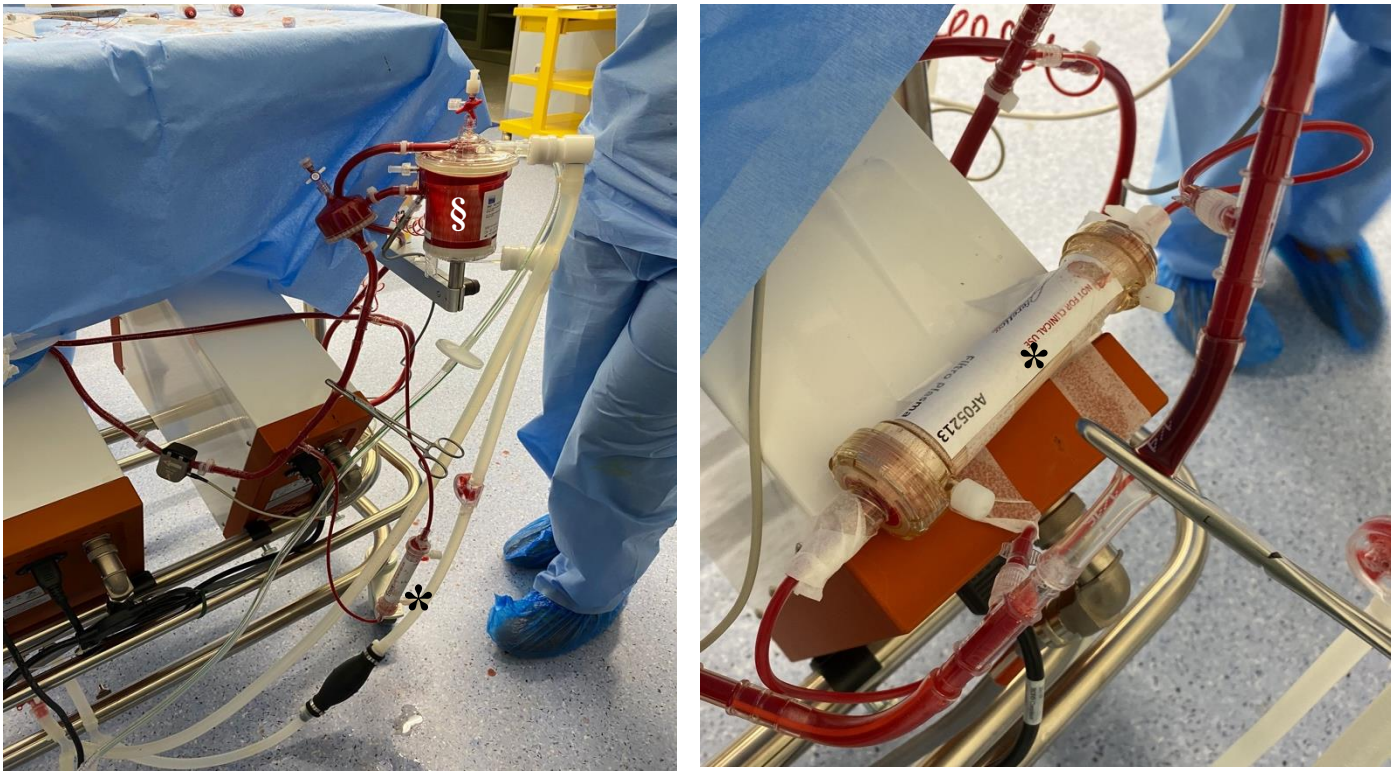
a Tissue NAD and NADH were evaluated in liver biopsies. The NMP+bioreactor group showed a greater amount of both total NAD and NADH content compared with the Native group ($p<0.0001$ and $p<0.0001$) and the standard NMP ($p<0.0001$ and $p=0.0007$). Bars denote mean±SEM, n=5 independent biological replicates. One way ANOVA, Tukey's post hoc test. p-value for group comparison: NAD total $p<0.0001$; NADH $p<0.0001$. Asterisks denote: ***$p<0.001$; **** $p<0.0001$. **b** NADH content was measured in perfusate samples troughout the NMP procedure. The NMP+bioreactor group showed higher NADH concentrations compared to the NMP group. Points denote mean±SEM, n=5 independent biological replicates. Two-way RM ANOVA, Tukey's post hoc test. p-value vs NMP: ***$p=0.001$. Abbreviations: b-MSCs, MSC-bioreactor; NAD, nicotinamide adenine dinucleotide; NMP, normothermic machine perfusion; W, wash-out.

Supplementary Figure 9. Plasmafilters used to implement the advanced NMP platform for large animals



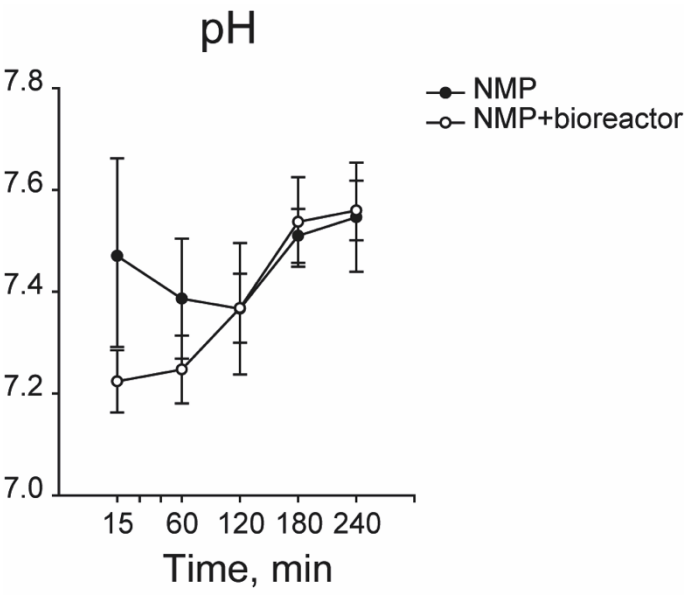
Human plasmafilters (Aferetica Srl) show a 2000 cm^2 surface area. Similar to the bioreactors used in the small animal study, the fibers are constituted from 150 nm pores-membranes.

Supplementary Figure 10. Connection of the human plasmafilter to the Liver Assist® perfusion platform.



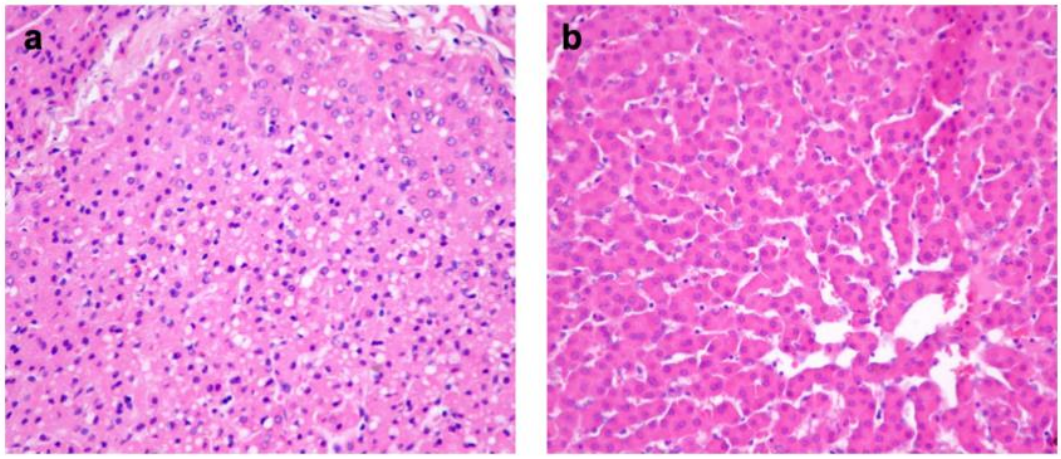
§ membrane lung oxygenator; * bioreactor connected to the main arterial circuit thorough a dedicated loop.

Supplementary Figure 11. Perfusate pH during porcine liver NMP



Points denote mean±SEM, n=3 independent biological replicates. Two-way RM ANOVA, Tukey's post hoc test. p-value for group comparison: p=0.233. Abbreviations: NMP, normothermic machine perfusion.

Supplementary Figure 12. Histological pictures of tissue biopsies collected before and after porcine liver NMP performed with the advanced platform



Representative images of morphological evaluations of liver tissue collected **a** before and **b** after the bioreactor-based perfusion procedure. **a** Absent necrosis and cholestasis, no sinusoidal dilatation (score 0); **b** Absent cholestasis (score 0), mild sinusoidal dilatation (score 1). Hematoxylin and eosin staining, original magnification: 20x

5. References

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