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# Contactless mechanical stimulation of tissue engineered constructs: development and validation of an air-pulse device.

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#### 10 1 Introduction

Tissue engineering (TE) is the study and development of biological substitutes to restore, maintain or improve tissue function [1,2]. TE is a multidisciplinary field that merges engineering and life science for the development of biocompatible and biomimetic tissue substitutes by using cells, growth factors and scaffolds. The ultimate goal of TE is to avoid the need to replace damaged tissue by stimulating or enhancing tissue repair and regeneration [3]. TE is motivated by three main health care issues, namely, the reliability of animal testing, the organ shortage crisis and drug development, which suffers from numerous failures during preclinical study phases (9 over 10) [4–6].

18 Despite over 30 years of research, tissue engineered constructs (TECs) still present differences in 19 mechanical and biological properties compared to native tissue [7-10]. The microstructure and mechanical 20 properties of biological tissues are mainly determined by the extracellular matrix (ECM). Mechanotransduction is 21 the process through which mechanical stimulation triggers proliferation, apoptosis, and ECM synthesis, among 22 other cell activities [11]. Regarding that aspect, the effect of in vitro stimulations such as compression, stretching, 23 bending or fluid shear stress loading modalities have been extensively studied [12]. Dynamic compressive or 24 stretching strain has been shown to induce gene expression related to the collagen metabolism involved in ECM 25 synthesis, especially in TECs based on fibroblastic cells [13-15], stromal cells [16] or mesenchymal stem cells 26 [17-20]. Regardless of the cell origin, to induce extracellular matrix synthesis, the mechanical stimulation 27 frequency is usually set to 1 Hz [13,15–17,21], which corresponds to the human walking frequency. In these 28 studies, the strain level ranged from 5 to 60% [13,15-17] depending on the scaffold and the cell type. It is 29 therefore paramount to be able to regulate the stimulation frequency and the strain level with respect to the TEC 30 studied.

31 To date, the devices used for these stimulations are largely represented by systems in direct contact with 32 the TEC, and there are few studies on "noncontact" stimuli [22,23]. A fluid flow used to produce noncontact 33 mechanical stimulation induced by an air pulse could be easily achieved in vivo without altering the tissue 34 integrity. In addition, in vivo, epithelial cells have apical access to ambient air, such as the intestinal or female 35 reproductive tract epithelium. Air-liquid interface cell cultures have been shown to be effective for the growth and 36 differentiation of cultured epithelia, such as corneal, nasal, middle ear, intestinal or gastric cells [24-26]. Air 37 exposure combined with mechanical stretch stimulation, mimicking the in vivo keratinization mechanism, has 38 been studied in a 3D culture system and provided encouraging results towards artificial skin substitutes [27]. 39 However, although air exposure and mechanical stress are the typical methods for cornification of skin 40 equivalents or keratinization, the effect of air as a mechanical stimulus medium for cell culture and, with greater 41 reason, for TEC maturation remains to be determined.

42 The objective of the study was to develop and validate a new air-pulse device for contactless and controlled 43 mechanical simulation of a TEC. Our hypothesis was that the development of a contactless stimulation device 44 with controlled mechanical parameters could be used during the maturation of scaffold-free TECs despite their 45 initial fragility. The device was designed to deliver an air pulse that is controlled, calibrated, and maintains the 46 sterility of the system within a noncytotoxic custom-made bioreactor. This study was conducted in the following 47 three phases: 1) conception of the controlled air-pulse device combined with a 3D printed bioreactor; 2) 48 experimental and numerical mechanical characterization of the air-pulse impact by digital image correlation (DIC); 49 and 3) achieving sterility and noncytotoxicity of the air-pulse and of the 3D printed bioreactor using a novel 50 dedicated sterilization process.

#### 51 2 Materials and methods

- 52 2.1 Conception of the controlled air-pulse device combined with a 3D printed bioreactor
- 53 2.1.1 Air-pulse control and monitoring

The schematic diagram of the air-pulse stimulation device is described in Figure 1. The air was extracted from the laboratory compressed air system and filtered through a Beko Clearpoint® (BEKO TECHNOLOGIES, Sarreguemines, France) air filter. The air was then regulated upstream with a pressure controller before being delivered through a servo-controlled solenoid valve. The air pulse was then separated into two streams to stimulate two TECs simultaneously.

59 The resulting volumetric flow rate and the pressure were measured for each stream using an air flow 60 metre SMC PFM7 (SMC Corporation, Tokyo, JAPAN) and a pressure sensor WIKA A-10 (WIKA, Klingenberg am 61 Main, Germany). The air-pulse was conducted through a flexible polyamide and polyurethane tube with a 4 mm 62 internal diameter and 1 m length and filtered once with a 0.2 μm filter before entering the incubator. The air flow 63 was filtered a second time 1 m downstream before being finally pulsed into the bioreactor and onto the TEC. An 64 acquisition card driven by a homemade program written with LabVIEW v2019 software was used to control and 65 monitor the air pulse.



66

67 *Figure 1: Schematic diagram of the air-pulse device. Air pressure is regulated (1). Then, the opening time of the solenoid* 68 *valve is controlled to deliver an air pulse (2) before being filtered and sterilized in the bioreactor (3).* 

#### 69 2.1.2 Air-pulse velocity at the nozzle outlet

The pipe lengths, the filters used to sterilize the air, and the delayed opening time of the solenoid valve influence the pressure and airflow losses from the input command to the nozzle outlet. An airflow metre and pressure sensor were used at the nozzle outlet to measure the maximum pressure and airflow losses during the pulse as a function of (i) the length, (ii) the pressure set, and (iii) the opening time of the solenoid valve to characterize the velocity of the air pulse at the nozzle outlet entering the bioreactor.

#### 75 2.1.3 3D printed bioreactor conception

76 A dedicated bioreactor (70x70x40 mm) was designed using SolidWorks v2019. The prototyping was constructed 77 with the use of a 3D printer (Ender 3 Max from CREALITY, Madrid, Spain) using fused deposition modelling (FDM) 78 technology with a 1.75 mm polylactic acid (PLA) filament from SUNLU (Guangdong, P.R. China). This biopolymer was 79 chosen because it is noncytotoxic and widely used for medical applications [28-30]. The 3D printed bioreactor consisted 80 of the following six different parts (Figure 2): the upper part, which includes inserts to plug the nozzle tip to hold the needle 81 or the sterile cap to ensure sterility; the lower part, which maintains the TEC; the overall sterility was maintained by an 82 autoclaved 20 mm diameter O-ring (BGS technic KG, Wermelskirchen, Germany) placed between the upper part and the 83 nozzle tip or the sterile cap.



Figure 2: Schematic of the 3D printed bioreactor. A. General overview. B. Cross-sectional view C-C: (1) upper part, (2) nozzle, (3) sterile
cap, (4) nozzle tip, (5) lower part, (6) O-ring.

87 2.2 Mechanical characterization of the air-pulse impact

88 2.2.1 Manufacture of the speckled silicone substitutes

89 LSR 4305 silicone (Elkem, Oslo, Norway) was used as a physico-chemical inert substitute for the TEC since it is 90 isotropic, homogeneous, and incompressible and since it does not dry out under an air pulse. This specific silicone was 91 chosen due to its Young's modulus, which is a similar that of in vivo tissues such as skin (222 kPa [31]) or cartilage 92 (varying from 0.02 to 6.44 MPa [32]). Components A and B (which contained the catalyst) were mixed 1:1 with 0.5% white 93 matt paint from a do-it-yourself store. The preparation was poured into a 30 cc syringe and centrifuged before being 94 weighed and poured into a 100 mm diameter petri dish to reach a height of 2 mm. After polymerization for 24 hours at 60 95 °C at a pressure of 700 mBar, light speckling by droplet deposition was performed on the surface using black aerosol paint 96 for further displacement field measurement by DIC. Samples of 15 mm diameter by 2 mm height were cut with a punch for 97 mechanical characterization of the substitute.

98 2.2.2 Mechanical characterization of the silicone substitute

99 Dynamic mechanical analysis (DMA) was performed on 4 samples of the silicone substitute using an oscillatory 100 compression test at 25 °C (DHR2, TA instrument, USA) [33]) with a 15 mm parallel plate geometry. This method was 101 chosen because it can also be performed on a TEC with a low elastic modulus (from a Pa to a few kPa).

102 The measurements of two oscillatory signals (force and displacement) provided the compression storage E' and 103 loss E'' moduli. First, the linear region was determined at a constant frequency (data not shown), then a frequency test 104 from 0.1 Hz to 10 Hz was performed at a constant strain (1%), which provided the linear viscoelastic region. The resulting 105 storage and loss moduli curves were fitted by a second-order generalized Maxwell model to extract the Young's modulus 106  $E_0$  and the overall viscosity  $\eta_0$ . This procedure is described more extensively in a previous work [34].

107 2.2.3 Surface displacement during air-pulse using digital image correlation (DIC)

108 The displacement field on the surface of the silicone substitute was measured during the application of the air-109 pulse by a DIC study, as described in Figure 3A. The air-pulse stimulation device was used to deliver air pulses controlled 110 by the LabVIEW interface via a nozzle held by a system designed and 3D printed specifically for the study.

Two synchronized high-speed cameras (SA3, Photron®, with an image size of 512 x 512 pixels), mounted with a pair of 105 mm lenses, were set up to record the area of interest (approximately 15 x 15 mm<sup>2</sup>) at a frame rate of 2000 images/s. Exposure time, aperture, and light intensity were chosen to obtain a good contrast and enough depth of field to ensure a good focus on the surface during the air-pulse application. Calibration of the setup and digital image correlation were performed using VIC3D software (VIC3D, version 8, Correlated Solution). The pixel size was approximately 0.05 mm. Computation was performed with a subset size of 23 pixels and a step of 5 pixels.

117 The 3D reconstruction of the silicone surface was obtained in a coordinate system for which the (x, y) plane was 118 tangent to the initial silicone plane. The results mainly focused on the altitude (*Z*) and the displacement normal to the 119 surface (*W*) of all points and the major Lagrange strain field.

To synchronize the recording of the images and the air-pulse, a transistor-transistor logic (TTL) signal was sent to trigger the recording of the cameras and to the air-pulse system acquisition card. 122 An image of the silicone surface before the air-pulse was applied was selected as the initial frame. During the 123 application of the air-pulse, the silicone surface was deformed in a well-shape, as described in Figure 3B. The point *J* was 124 defined as the well bottom. During the stabilized phase of a given air pulse, its location in the (x, y) plane was stable. An 125 average stimulation frame was selected in this stabilized phase to analyse the well shape. Twenty depth profiles were 126 extracted from planes  $\mathcal{P}_i(J, \vec{u}_i, \vec{z})$ , where  $\vec{u}_i = \cos \theta_i \vec{x} + \sin \theta_i \vec{y}$ ,  $\theta_i = \frac{\pi i}{20}$  and  $i \in [1, 20]$ . After  $\theta_i$  clockwise rotation, these 127 profiles were plotted in the same plane (x, z).



128

Figure 3: DIC study. A. Schematic diagram of the DIC experiment. B. Graphical representation of the methodology followed to extract 20 depth profiles.

#### 131 2.2.4 Numerical simulation of the air-pulse mechanical stimulation

To assess the mechanical effect of the air pulse on a TEC, a numerical simulation was developed in Comsol Multiphysics (version 5.3, COMSOL, Inc.). A fully coupled fluid-structure interaction (FSI) approach was used to model the impact of the air-pulse on the TEC represented by the silicone substitute. Due to the axial symmetry of the problem and to enhance computation time, axisymmetric modelling was used. A stationary analysis was performed to compare the numerical surface displacement profile of the substitute induced by a stabilized volumetric air flow rate to the DIC data acquired at a stabilized surface displacement profile.

138 The model described in Figure 4 is composed of (i) a fluid domain D1, which represents the air with a box of 20 139 mm radius (in axisymmetric representation) and 36 mm height, with material properties from the Comsol toolbox and (ii) a 140 solid domain D2, which represents the silicone substitute with a box of 15 mm radius (in axisymmetric representation) and 141 2 mm height with material properties determined from DMA. The nozzle is represented by its boundary conditions BC1 142 (inlet mass flow rate,  $Q_{sv} = 2.1e^{-4} m^3/s$  experimentally characterized) and BC3 (inner wall with no-slip boundary 143 condition) with a 2 mm inlet radius for a 8 mm height and a 0.5 mm outlet radius for a 14 mm height. The computed 144 domain is closed by a fluid boundary condition BC2 (atmospheric pressure) and a wall BC4 with no slip condition, a flat 145 support condition BC5 ( $U \cdot e_z = 0$ ) and an embedding BC6 (U = 0) at the point r = z = 0 to prevent rigid body motion, 146 with U being the solid displacement. The fluid-structure interaction is described by a fully coupled interface BC7. A finer 147 physics-controlled mesh generated on the geometry by Comsol was used and provided a total of 26°293 triangle 148 elements.

149 For the fluid domain (D1), the Reynolds and Mach numbers were computed to design the numerical study, which were  $Re = \frac{Q_{sv} \times d \times \rho}{s * \mu} = 2.56e^4$  and  $Ma = \frac{Q_{sv}}{s \times a} = 1,12$ , respectively, where d = 1 mm is the diameter of the nozzle outlet, 150  $S = 0.8 mm^2$  is the surface of the nozzle outlet,  $\mu = 1.849e^{-5} \text{ kg/m.s}$  is the dynamic viscosity of the fluid,  $\rho = 1.184 \text{ kg/m}^3$ 151 152 is the density of the fluid and a = 343 m/s is the speed of sound in air. The Reynolds Averaged Navier–Stokes (RANS)-153 based turbulence models were used to describe the fluid domain of a stationary compressible mean flow (Eq. 1), where  $\rho_f$ 154 is the fluid density, p is the mean pressure, v is the fluid velocity vector,  $\mu$  is the dynamic viscosity,  $\mu_T$  is the turbulent 155 viscosity, K is the viscous stress tensor, F is the external forces applied to the fluid and I is the identity tensor. The 156 accuracies of the two-equation turbulence models (k- $\epsilon$  and k- $\omega$ ) were compared for the prediction in the impinging jet 157 modelling [35,36]. The standard two-equation k-ɛ model was then chosen, as illustrated in the results and discussion 158 sections.

$$\rho_f(\boldsymbol{v} \cdot \nabla \boldsymbol{v}) = \nabla \cdot [-p\boldsymbol{I} + \boldsymbol{K}] + \boldsymbol{F}; \quad \nabla \cdot (\rho_f \boldsymbol{v}) = 0;$$

$$\boldsymbol{K} = (\mu + \mu_T) \left( \nabla \boldsymbol{v} + (\nabla \boldsymbol{v})^T - \frac{2}{3} (\nabla \cdot \boldsymbol{v}) \boldsymbol{I} \right)$$
Eq. 1

159 For the solid domain (D2), experimental tensile tests on the silicone performed for a strain <100% (data not 160 shown) in two perpendicular directions demonstrated a linear behaviour. Moreover, the viscous behaviour was considered 161 negligible because of its very low overall viscosity  $\eta_0$ , as described in the results section. Therefore, the silicone substitute 162 was modelled as an isotropic linear elastic material using Hooke's law with elastic modulus E=210 kPa measured 163 experimentally using DMA (cf. results section), Poisson's ratio = 0.49 (to enable numerical convergence instead of 0.5 164 classically used for elastomers) and density  $\rho_s = 1.09$  g. cm - 3 (from the manufacturer data sheet). Elastostatic equations were used to solve the solid part (Eq. 2), with  $\sigma$  as the Cauchy stress tensor,  $f_V$  as the volume force vector,  $\mathbb C$  as the 165 stiffness tensor,  $\varepsilon$  as the small strain tensor and u as the solid displacement vector. 166



167

Figure 4: Mesh and boundary conditions of the axisymmetric numerical model. The fluid domain (D1), which represents the air, is in
 green blue. The solid domain (D2), which represents the TEC (silicone substitute), is in red. BC1 is the inlet condition (Q =

170  $2.1e^{-4} m^3/s$ ), BC2 is the outlet boundary condition ( $P = P_{atm}$ ), BC3 is the inner wall boundary condition (no-slip boundary

171 condition), BC4 is a wall with slip condition, BC5 is the flat support condition, BC6 is the solid embedding point and BC7 is the fluid
 172 structure interaction interface (fully coupled).

#### 173 2.2.5 Validation of the numerical model

The numerical results were compared to the vertical surface displacement profiles of the silicone measured on 20 cutting planes from 0 to 180° by DIC (Figure 6). First, over the radial interval r = [-4;4] mm, the experimental and numerical displacements, i.e.,  $W^{exp}$  and  $W^{num}$ , respectively, were shifted so that  $\min(|W^{exp}|) = \min(|W^{num}|) = 0$ . Since the radial coordinates  $r^{exp}$  differed between each experimental cutting plane, linear interpolation using a first-order Taylor formula was performed to compute the interpolated experimental displacement  $W^{int}$  for each cutting plane to plot them within the same radial interval  $r^{num} = [-4; 4; 0.1]$ , as described in Eq. 3.

$$\forall j \in [-4; 4; 0.1] \& k \in [r^{exp}] \mid (k-1) < j \& (k+1) > j,$$

$$W_j^{int} = W_{k-1}^{exp} + (r_j^{num} - r_{k-1}^{exp}) \times \frac{W_{k+1}^{exp} - W_{k-1}^{exp}}{r_{k+1}^{exp} - r_{k-1}^{exp}}$$
Eq. 3

180

181 Second, the mean interpolated experimental displacement profile  $\overline{W^{int}} = \frac{\sum_{i=1}^{20} w_i^{int}}{20}$  was computed as the average of the 20 cutting planes.

183 Third, the comparison between the mean experimental displacement profile and the simulated one was performed using 184 the coefficient of determination calculation described in Eq. 4 and by the difference between the area under the two curves 185 using the rectangle method.

$$\forall j \in [-4; 4; 0.1], \quad R^{2} = \frac{\sum_{j=1} (W_{j}^{num} - \overline{W^{int}})^{2} - \sum_{j=1} (W_{j}^{num} - W_{j}^{int})^{2}}{\sum_{j=1} (W_{j}^{num} - \overline{W^{int}})^{2}} \qquad \qquad Eq. \ 4$$

#### 186 2.3. Sterility and noncytotoxicity of the device

#### 187 2.3.1. Air-pulse sterility

188 The sterility of the air-pulsed through the nozzle was characterized. The air-pulse device was used to perform an air-189 pulse stimulation test under sterile conditions in a complete cell culture medium composed of 10% FBS (foetal bovine serum), 1% penicillin/streptomycin, 0.1% amphotericin B and DMEM Glutamax (Thermo Fisher Scientific, Waltham, 190 191 Massachusetts, USA). The device was placed inside the incubator at 37 °C and 5% CO2 and the air-pulse stimulations 192 occurred for 10 minutes once a day for 3 days and the device was left further for one week without stimulations. The cell 193 culture medium was then collected and subjected to bacteriological analysis in the bacteriological Analysis Veterinary 194 Laboratory (LAV, Marcy l'Etoile, France) through Gram staining, aerobic culture (Columbia blood agar with sheep blood 195 medium, BioMérieux, Marcy l'Etoile, France), TSB (Tryptic Soy Broth, Bio-Rad, Hercules, California, USA) and 196 thioglycolate broth (THIO-CLAIR, BioMérieux, Marcy l'Etoile, France) to assess the absence of contamination.

#### 197 2.3.2. 3D printed bioreactor noncytotoxicity and sterility

198 The noncytotoxicity of the bioreactor was ensured using PLA, a biopolymer obtained from the fermentation of corn 199 starch under the effect of bacteria producing lactic acid and used as a 3D printing material in FDM technology.

A dedicated homemade sterilization protocol for the printed bioreactor was developed. Since PLA has a glass transition temperature tg = 60 °C, the 3D printed bioreactor was immersed in a 70° ethanol bath for 72 hours and then autoclaved at 105 °C for 25 minutes. As the cytotoxicity of the printed Sunlu PLA+ material after thermochemical treatment has never been assessed, biological evaluations were needed. The study of the cytotoxicity of the autoclaved bioreactor was performed based on the ISO 10993-5 standard for *in vitro* cytotoxicity, as follows.

205 Mouse fibroblast cells from an L929 cell line were cultured in contact with the material for 72 hours. The L929 cell 206 line was purchased from Sigma (Saint Louis, Missouri, USA) and used for the cytotoxicity assay of the printed material. 207 Cells were thawed and seeded at 2500 cells/cm<sup>2</sup> in a T75 flask in a complete cell culture medium composed of 10% FBS 208 (foetal bovine serum), 1% penicillin/streptomycin, 0.1% amphotericin B and DMEM Glutamax (Thermo Fisher Scientific, 209 Waltham, Massachusetts, USA). The cells were incubated at 37 °C in a 95% humidified atmosphere (5% CO2). After 7 210 days of culture, the cells were harvested following a classic protocol with 5% EDTA trypsin and resuspended in a complete 211 cell culture medium. A proliferation assay was previously performed for 3 days in a 6-well plate with varying concentrations 212 of cells to obtain the optimum confluence of the cell monolayer. The cells were seeded at 2500 cells/cm<sup>2</sup> for the 3 groups-213 test, as follows: (1st group named: Control) a blank control where cells were seeded in a regular culture dish, (2nd group 214 named: PLA) a direct contact where cells were seeded in a 3D printed PLA culture dish, and (3rd group named: Latex) a 215 direct contact cytotoxic positive control where cells were seeded in a regular culture dish with a latex sample. All test 216 conditions were performed three times in triplicate in 9.6 cm<sup>2</sup> wells (6-well plate) for 72 hours before the cells were 217 trypsinized and enumerated with trypan blue. A BD Pharmingen apoptosis and mortality detection kit ANNEXIN V 7AAD 218 was then used according to the manufacturer's instructions [37-39]. The cells were resuspended at a concentration of 10<sup>6</sup> 219 cells/ml in a binding buffer solution. One hundred microlitres of this solution was drawn, and 5 µL each of ANNEXIN V and 220 7AAD staining solution was added. The cells were then analysed using a BD Accuri C6 Plus flow cytometry system to 221 determine the cellular viability and, thus, determine the cytotoxicity of PLA. Cell apoptosis and mortality were analysed by 222 Annexin V staining, and apoptosis was analysed by 7AAD staining. The morphology and granularity of the cells were 223 analysed by flow cytometry. The data were collected upon 15,000 events per sample.

224 Statistical analysis was performed in R (R Foundation for Statistical Computing, Vienna, Austria), normality was 225 checked using the Shapiro–Wilk normality test, and equal variance was checked using the Bartlett test. After assessment 226 of normality of distributions and equal variance, a one-way ANOVA was performed with Tukey HSD as post hoc tests.

#### 227 3. Results

#### 228 3.1. Air-pulse monitoring

Figure 5 shows that the volumetric flow rate of the air-pulse measured at the output of the nozzle quickly stabilized after 100 ms for a set pressure of 2 bar and an opening time of the solenoid of 1 s. The value of  $4.1e^{-4}$  m<sup>3</sup>/s corresponding to the mean value of the plateau of the volumetric air flow rate curve was used as the inlet condition at the nozzle input for the numerical simulation.



Figure 5: Volumetric air flow rate measured at the output of the nozzle for a set pressure of 2 bar and an opening time of the solenoid
 of 1 s.

236 3.2. Mechanical characterization of the air-pulse impact

237 3.2.1. Mechanical characterization of the silicone substitute

The elastic contribution (storage modulus) and viscous contribution (loss modulus) of the silicone were characterized by DMA (Figure 6A) as a function of the loading frequency, as illustrated in Figure 6B. Figure 6C displays the computed Young's modulus  $E_0 = 210$  kPa and overall viscosity  $\eta_0 = 1.7 e^{-4}$  kPa.s obtained for the 4 samples characterized. The ratio between the mean viscosity and Young's modulus  $E_0/\eta_0 = 1.23 e^6 s^{-1}$  leads to ignoring the viscous contribution of the material. Therefore, the behaviour of the silicone substitute was modelled as a purely linear, isotropic and homogeneous elastic material with E = 210 kPa, v = 0.49 and  $\rho_s = 1.07$  g.  $cm^{-3}$  [40].

244



Figure 6: Results of the dynamic mechanical analysis: A. DMA system with a sample represented in pink; B. experimental data (dotted lines) and fitted numerical results (solid lines) of the storage modulus (red) and the loss modulus (green); C. mean values of the mechanical parameters computed for the 4 silicone samples.

#### 249 3.2.2. Surface displacement during air-pulse impact using DIC

250 The different air pulses applied on the silicone surface are clearly visible in the normal displacement W for the 251 well-bottom J, always reaching the same depth under pressure, and its location in the plane (x, y) remains stable. 252 However, it did not recover its initial position between the different air pulses ( $17.2 \pm 0.4 \mu m$  difference between 5 pulses). 253 The 3D reconstruction of the silicone surface corresponding to the initial frame and the average stimulation frame are 254 given in Figure 7. Figure 7A shows that the silicon surface before the air pulse is applied is not perfectly plane in the 255 impact zone (magnitude of  $134 \pm 8 \mu m$  between the extrema). At the average stimulation frame, the point *I* defined at the 256 well bottom moved  $190.1 \pm 2.9 \,\mu$ m from the initial frame. However, the effect of the air-pulse was not axisymmetric on the 257 Z profile; thus, an ellipse was indeed predicted at the air-pulse impact, as depicted in Figure 7B.

Major Lagrange strain distributions are mapped on the 3D silicone surface in Figure 7C. The major and minor strain values did not exceed 1.28% and 0.06%, respectively, and the maxima did not coincide with the well bottom *J*. A vector field of the major strains is also provided in Figure 7D, as indicated by white arrows superimposed on a *Z* map of the silicone surface. The vector field described an ellipse, and its focus seemed to be approximately at the well bottom position.



263

Figure 7: Surface deformation of the silicon substitute under an air pulse. A & B. Surface altitude (Z) distribution: A. before air-pulse
 stimulation (initial frame) and B. under air-pulse stimulation (average stimulation frame). C. Major Lagrange strain distribution under
 air-pulse stimulation. D. Vector field of major Lagrange strains (white arrows) superimposed on the altitude (Z) distribution.

267 3.2.3. Numerical simulation of the air-pulse mechanical stimulation

268 The calculation converged in 86 seconds on a laptop (I7 processor with 2,6 GHz and 16 Go of RAM), and the 269 segregated solver displayed error values for velocity, pressure, space referential coordinates, turbulence and displacement fields in the range of 10e<sup>-4</sup> and 10e<sup>-5</sup>, as recommended to conclude on the convergence of the model [41]. 270 271 The convergence of the model was also verified by the mass conservation study, and the calculated error between the 272 ratio of the total mass flow and the output of the model was  $e_{num} = 2\%$ , which is below the relative tolerance  $e_{tol} = 5\%$ 273 (data not shown). The mesh convergence was studied by checking the maximal numerical displacement normalized by 274 the maximal experimental displacement measured by DIC as a function of the number of elements. The model quickly 275 converged to 1 for the k-ε turbulence model, as shown in Figure 8. The k-ω turbulence model diverged and reached a 276 plateau with a maximum surface displacement four times greater than the experimental displacement. A simulation with a 277 finer physics-controlled mesh made of 26°293 triangle elements with the k-ɛ turbulence model was therefore used for the 278 comparison of the surface displacement profiles between the simulation and the DIC results, as illustrated in the next 279 section.





281 Figure 8: Mesh convergence study performed by checking the maximum surface displacement of the simulation normalized by the 282 maximum experimental displacement value for the k- $\omega$  (red square) and k- $\varepsilon$  (green triangle) turbulence models.

The simulated velocity profile along the axis of symmetry from the nozzle inlet to the surface of the silicone is 284 illustrated in Figure 9Figure 1A, and the iso-value air flow velocity map is depicted in Figure 9B. The simulation gives a 285 maximal velocity of  $v \cdot e_z = 460 \text{ m. s}^{-1}$  inside the nozzle, which decreases drastically to a velocity of  $11.2 \text{ m. s}^{-1}$  at the 286 point of impact with the surface of the silicone substitute. The maximum displacement of the silicone surface due to the air 287 flow was W = 0.177 mm (raw data without shifting). A negative pressure gradient is observed along the surface of the 288 silicone substitute during stationary flow.



289

290 Figure 9: Numerical results of the fluid structure interaction study in stationary mode: A. air velocity profile (m/s) along the axis of 291 symmetry, from the nozzle inlet to the silicone substitute surface; B. computed axisymmetric simulation of the fluid structure 292 interaction; the contour of the fluid pressure is displaced from 0 to -100 Pa; C. surface displacement profile (mm) of the silicone 293 substitute from the impact point the to edge.

#### 294 3.2.4. Comparison of the experimental and numerical surface displacement profiles

295 The surface displacement profiles of the 20 cutting planes measured experimentally by DIC over a 4 mm radius 296 around the centre of impact are depicted with the numerical profile in Figure 10. The numerical profile (red line) is very 297 similar to the averaged experimental profile (black dashed line). The difference between the absolute maximum 298 displacements of the averaged experimental data (0.156 mm) and the numerical result (0.155 mm) is 0.6%.

299 The coefficient of determination between the averaged experimental data and the numerical result of the surface 300 displacement (black dashed line and red line, respectively) was R<sup>2</sup> = 0.98. The comparison of the area under the curve 301 between these two profiles showed a difference of 4% over the interval r = [-2;2].



Radial coordinate along silicon surface [mm]

302

303 Figure 10: Comparison of the vertical displacement profiles along the surface of the silicone under an air pulse for the experimental 304 data (20 cutting lines represented by grey dotted lines with the overall trend in cyan and the averaged curve, represented by the black 305 dashed line) and the numerical result (red line). The experimental curves are not symmetric, which explains why they seemed to be 306 below the numerical curve, and the minimum of the averaged experimental curve is different from zero.

307 3.3. Sterility and noncytotoxicity of the device

#### 308 3.3.1. Air-pulse sterility

309 After one week, gross visual inspection of the phenol red in the medium as well as the microscopic observations 310 showed no apparent contamination or change in pH of the medium for both the control and air-pulse samples. Further 311 investigation with Gram staining and aerobic, aerobic TSB and anaerobic thioglycolate broth culture tests confirmed the 312 absence of contamination, as summarized in Table 1.

313

	Gram staining	Aerobic culture	Aerobic TSB	Anaerobic thioglycolate broth
Air-pulse stimulation	Negative	Negative	Negative	Negative
Control	Negative	Negative	Negative	Negative

Table 1: Sterility analysis of the L929 cell line seeded at 2500 cells/cm<sup>2</sup> by Gram staining, aerobic culture, TSB (tryptic soy broth) and 314 315 thioglycolate broth. The cells were stimulated for 3 days, 10 minutes once a day and incubated at 37 °C and 5% CO2 for a total 316 incubation time of 7 days.

317 3.3.2. Noncytotoxicity of the autoclaved 3D printed bioreactor

318 After 72 hours of culture, flow cytometry analysis of living and nonapoptotic cells showed a significant difference 319 (p-value < 0.0001) between Latex (cytotoxic positive control group) and PLA as well as Control (negative control group), 320 as shown in Figure 11. There was no significant difference between PLA and Control.



321

Figure 11: Box plot of flow cytometry results of the cytotoxicity tests of the PLA. PLA group in direct contact was compared with the positive control (Latex) and the negative control (Control) for living and nonapoptotic L929 cells initially seeded at 2500 cells/cm<sup>2</sup>.
 \*\*\*\*: p- value < 0.0001. The cells were incubated for 72 hours at 37 °C and 5% CO2.</li>

#### 326 4. Discussion

327 The study was primarily focused on the development of an air-pulse device prior to its use on TEC for a long-term 328 goal of using air-pulse stimulation for in vivo applications (e.g., epithelia). Our hypothesis was that the development of a 329 noncytotoxic contactless stimulation device with controlled mechanical parameters could make it possible to achieve 330 effective and noninvasive mechanical stimulation of TECs. To be able to do so, a controlled air-pulse device was first 331 developed. Then, we characterized the mechanical stimulation of the air-pulse impact both experimentally and 332 numerically. Finally, the sterility of the air pulse and the noncytotoxicity of the homemade 3D-printed bioreactor were 333 assessed. The developed numerical model will make it possible to further estimate the micromechanical phenomenon 334 induced by air-pulse stimulation of a TEC, which are impossible to directly measure, leading to changes in cellular activity 335 during maturation. Additionally, the simulation could be used to estimate and adjust the air-pulse parameters with respect 336 to the evolution of the TEC's mechanical properties during maturation to achieve the desired surface displacement profile 337 of the TEC.

338 Sterility and noncytotoxicity of the device must be strictly assured to avoid any contamination and perturbation in 339 cellular responses for further use with TECs. Regarding the sterility of the air-pulse (Table 1), the results showed that 340 there was no contamination in a 3-day time period of experimentation, which illustrates a well-designed stimulation work-341 flow, as illustrated in Figure 1. However, since the developed device was not directly placed in an incubator, contamination 342 due to repetitive manipulation of the bioreactor is inevitable if strict sterile conditions are not respected by the operator.

343 PLA is widely used for prototyping by 3D printing and has been demonstrated to be biocompatible and 344 noncytotoxic [28-30] in addition to being biodegradable [28]. A novel sterilization process for PLA was developed in this 345 study based on a thermochemical process. We demonstrated that the treated PLA was noncytotoxic and did not influence 346 cell proliferation (Figure 11). Furthermore, the initial shape of the 3D-printed bioreactor was preserved (data not shown). 347 The absence of cytotoxicity due to the sterilization process might be due to an additional esterification reaction during the 348 autoclave of the PLA at 105 °C for 25 minutes [42]. This reaction is probably caused by the immersion in the 70° ethanol 349 bath for 72 hours, which finalizes the cross-linking of the polymer and preserves the initial shape. To confirm the effect of 350 the chemical reaction of the ethanol bath on the PLA, a specific study should be performed to fully characterize the 351 chemical phenomenon that preserves the geometry of the 3D printed object after autoclaved sterilization. Additionally, 352 since the maturation period can be longer than 14 days for TEC maturation [19,43,44], further noncytotoxicity tests over a

353 longer period should be performed. In addition, further investigations such as proteomic profiling including chemokines 354 and cytokines profiling, should also be conducted to verify that cellular metabolism is not impaired, in order to assess the 355 biocompatibility of PLA according to the dedicated application [45,46].

A silicone material (LSR 4305) was used to characterize the mechanical behaviour of the air-pulse impact because of the stability over time of its mechanical properties [47,48]. DMA tests were performed to characterize the viscoelastic properties of the silicone, concluding that the viscous part can be neglected regarding the elastic part for frequencies within the range of 0.1 to 10 Hz, which corresponds to the mechanical stimulation frequency of the air-pulse, as discussed below.

The surface displacement of the silicone substitute induced by the air pulse was characterized by DIC. Since the viscous part was negligible, to prevent oscillatory effects of the silicone surface induced by a short air pulse, which could complicate the DIC analysis, a stabilized air flow at the nozzle outlet was achieved by opening the solenoid valve for a long time period [49]. A solenoid valve opening timespan of 1000 *ms* was chosen, which is 10 times higher than the air flow rate stabilization time (Figure 5). The major Lagrangian strain values of the silicone surface computed from DIC did not exceed 1.28% (Figure 7C). These results indicate that the silicone substitute under the air-pulse behaves as a perfectly linear elastic material and was modelled as such in the numerical simulation.

Experimentally, between each air pulse, a mean vertical residual displacement with respect to the initial configuration state was observed. This partial displacement recovery could be explained by the adhesion of the silicone on its support in the deformed configuration state, preventing a complete return to its initial (undeformed) configuration state. This partial displacement recovery (which represents 9% of the maximum displacement) should be prevented in immersion conditions corresponding to the real situation for TEC maturation, where DIC might not be possible due to important rigid body motion and cell culture medium refractive index and colour altering the image correlation.

The mean value of the raw maximum displacement of the 20 silicone surface displacement profiles analysed every 9° from 0 to 180° during a pulse was  $0.190 \pm 9.7e^{-5}mm$  (data not shown). The areas under the curves were calculated for each of the 20 experimental profiles, and the difference between each profile varied from 0.05% to 8.2%. The lack of axial symmetry, also illustrated by the elliptical shape of the impact observed on the surface displacement 2D map (Figure 7B), cannot be explained by the anisotropic behaviour of silicone, as conventional silicone materials are isotropic, but it can be explained by the nonnormality of the nozzle with the silicone surface during the pulse and the nonplanarity of the initial surface.

381 For high Reynolds numbers, Reynolds-averaged Navier-Stokes (RANS)-based turbulence models can be used 382 to model a fluid exiting a nozzle on a wall, called an impinging jet, to describe the heat transfer phenomenon [35,36]. 383 Impinging jets are schemed by different regions from the outlet to the wall, as follows: core region, developing region, fully 384 developed region, stagnation region and wall jet region. Each region designates a specific fluid-flow profile where physical 385 phenomena are better described in regard to the chosen turbulence model. To the best of our knowledge, very few studies 386 have dealt with impinging jets on a deformable wall using FSI. Direct numerical simulations, large eddy simulations (LESs) 387 or even hybrid RANS/LESs would be the most suitable numerical methods to describe the complex FSI involved in 388 impinging jets on a deformable wall. However, those methods are very difficult to implement and are very costly in terms of 389 the computation time needed. Additionally, in this study, we were only interested in the averaged displacement profile of 390 the solid part and were not concerned with the effect of the solid part on the fluid velocity. Therefore, we have chosen the 391 most commonly used two-equation turbulence models for engineering problems, which have been shown to well describe 392 shear stress and pressure at the wall [35]. In this study, the two-equation turbulence models (k-ε and k-ω) were compared 393 to assess the computed solid displacement profile against experimental data from DIC. The maximum surface 394 displacement in the k-s turbulence model converges rapidly to the experimental value (Figure 8). The k-w turbulence 395 model overestimates the experimental values. These results show that the k-ɛ turbulence model is better able to describe 396 the solid displacement profile induced by an impinging jet with a low Reynolds number on a deformable body with an elastic modulus on the order of hundreds of kPa. Further investigations could be performed to study the relation between 397 398 the air flow velocity and the solid displacement profile under immersed conditions in a cell culture medium corresponding 399 to the real situation for TEC maturation. Additionally, a wider range of elastic and visco-elastic properties should be 400 investigated to consider the variability of TEC mechanical behaviours.

401 The raw numerical surface displacement profile showed a maximum of 0.177 mm at the stagnation point (arc 402 length = 0) and an overall minimum of 0.016 mm at the right edge of the solid part compared to the initial surface 403 configuration in the steady state (Figure 9C). The nonnull displacement along the whole solid surface and especially at the 404 right edge can be explained by a nonnull pressure induced by the stationary flow along the surface of the solid [50], as 405 depicted by the pressure contour lines in Figure 9B. Therefore, to compare the numerical to the experimental displacement profiles, their minimums were adjusted such that  $\min(|W_i^{exp}|) = \min(|W^{num}|) = 0$  (where  $i \in [1,20]$ 406 407 represents each experimental silicone surface displacement profile line cf. Figure 3B), and the entire curves were shifted 408 accordingly. The comparison between the numerical and averaged experimental profiles (red line and black dashed line, 409 respectively, as depicted in Figure 10) gives a coefficient of determination  $R^2 = 0.98$  and a difference of 4% between the 410 areas under the curves. From these results, it can be concluded that the numerical simulation provides a predictable 411 surface displacement profile. Therefore, the numerical simulation could be used to study the micromechanical effects412 induced by an air pulse and to adjust the air flow rate to the maximum surface displacement desired on a TEC.

## 413 5. Conclusion

414 This study led to the development and characterization of a calibrated air-pulse device for TEC mechanical 415 stimulation during maturation. To fully decipher the mechanical stimulation induced by an air pulse, it was characterized by 416 DIC, and a numerical twin of the device was developed. Since the mechanical properties of the TEC evolve during the maturation process, the numerical model allows us to adjust the air-pulse parameters according to the desired surface 417 418 displacement of the TEC. In addition, the numerical model can be used to investigate the micromechanical effects of air 419 pulses inside the TEC, which cannot all be measured experimentally, for instance, wave propagation generated during the 420 air-pulse impact. Linking the biological response and the micromechanical effects generated during air-pulse stimulation 421 will improve our understanding of the cellular response to mechanical stimulation. An ethanol/autoclaved sterilization 422 protocol for 3D printed objects in PLA has been developed in this study, enabling the use of 3D printing in cell culture. 423 Further studies remain to be conducted to ensure that PLA is noncytotoxic and that the incubator environment does not 424 affect its properties over periods of more than one month related to the characteristics of TEC maturation. The device can 425 be used to study the cell response to contactless cyclic mechanical stimulation, particularly in TECs with fibroblasts, 426 stromal cells and mesenchymal stem cells, which have been shown to be sensitive to the frequency and strain level at the 427 air-liquid interface.

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