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Biocompatibility and Immunogenicity of Decellularised Allogeneic Aorta in the Orthotopic Rat Model

Michalis Katsimpoulas^{1,2*}, Lucrezia Morticelli^{3*}, Ioanna Gontika⁴, Artemis Kouvaka³, Panagiotis Mallis⁴, Daniele Dipresa³, Ulrike Böer³, Bisharah Soudah⁵, Axel Haverich^{3,6}, Efstathios Michalopoulos⁴, Sotiris Korossis^{3,6,7}

1. Centre of Clinical, Experimental Surgery and Translational Research, Biomedical Research Foundation of the Academy of Athens, 4 Soranou Ephesus Street, 11527 Athens, Greece;

2. Attikon Animal Hospital, Lavriou Av., 19002 Paiania, Greece;

3. Lower Saxony Centre for Biomedical Engineering Implant Research and Development, Hannover Medical School, Stadtfeldamm 34, 30625 Hannover, Germany;

4. Hellenic Cord Blood Bank, Biomedical Research Foundation Academy of Athens, 4 Soranou Ephesus Street, 11527 Athens, Greece;

5. Institute for Pathology, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany;

6. Department of Cardiothoracic, Transplantation and Vascular Surgery, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany;

7. Centre for Biological Engineering, Wolfson School of Mechanical, Electrical and Manufacturing Engineering, Loughborough University, Asby Road, LE11 3TU Loughborough, United Kingdom.

* Contributed equally to this work as first authors

Corresponding Author

Michalis Katsimpoulas, DVM, SRS, PhD

Center of Clinical, Experimental Surgery and Translational Research, Biomedical Research Foundation of the Academy of Athens, 4 Soranou Ephesus Street, 11527 Athens, Greece

T: +30 210 6597367

F: +30 210 6597365

E-mail: mkatsiboulas@bioacademy.gr

Lucrezia Morticelli, PhD

Lower Saxony Centre for Biomedical Engineering Implant Research and Development, Department of Cardiothoracic, Transplantation and Vascular Surgery, Hannover Medical School, Stadtfeldamm 34, 30625 Hannover, Germany

T: +49 511 5328422

F: +49 511 5328314

Email: morticelli.lucrezia@mh-hannover.de

Ioanna Gontika, MSc

Hellenic Cord Blood Bank, Biomedical Research Foundation of the Academy of Athens, 4 Soranou Ephesus Street, 11527 Athens, Greece

T: +30 210 6597331

39 F: +30 210 6597342

40 E-mail: igontika@bioacademy.gr

41

42 Artemis Kouvaka, PhD

43 Lower Saxony Centre for Biomedical Engineering Implant Research and Development, Department of Cardiothoracic,
44 Transplantation and Vascular Surgery, Hannover Medical School, Stadtfelddamm 34, 30625 Hannover, Germany

45 T: +49 511 5327797

46 F: +49 511 5328314

47 Email: kouvaka.artemis@mh-hannover.de

48

49 Panagiotis Mallis, MSc

50 Hellenic Cord Blood Bank, Biomedical Research Foundation of the Academy of Athens, 4 Soranou Ephesius Street,
51 11527 Athens, Greece

52 T: +30 210 6597331

53 F: +30 210 6597342

54 E-mail: pmallis@bioacademy.gr

55

56 Daniele Dipresa, PhD

57 Lower Saxony Centre for Biomedical Engineering Implant Research and Development, Department of Cardiothoracic,
58 Transplantation and Vascular Surgery, Hannover Medical School, Stadtfelddamm 34, 30625 Hannover, Germany

59 T: +49 511 5327797

60 F: +49 511 5328314

61 Email: dipresa.daniele@mh-hannover.de

62

63 Ulrike Böer, PhD

64 Lower Saxony Centre for Biomedical Engineering Implant Research and Development, Department of Cardiothoracic,
65 Transplantation and Vascular Surgery, Hannover Medical School, Stadtfelddamm 34, 30625 Hannover, Germany

66 T: +49 511 5321453

67 F: +49 511 5328314

68 Email: boeer.ulrike@mh-hannover.de

69

70 Bisharah Soudah, PhD

71 Institute for Pathology, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany

72 T: +49 511 5324512

73 F: +49 511 5325799

74 Email: soudah.bisharah@mh-hannover.de

75

76 Axel Haverich, Dr.h.c.

77 Department of Cardiothoracic, Transplantation and Vascular Surgery, Hannover Medical School, Carl-Neuberg-Str. 1,
78 30625 Hannover, Germany
79 T: +49 511 5326580
80 F: +49 511 5325404
81 Email: haverich.axel@mh-hannover.de

82
83 Efstathios Michalopoulos, PhD
84 Hellenic Cord Blood Bank, Biomedical Research Foundation of the Academy of Athens, 4 Soranou Ephesius Street,
85 11527 Athens, Greece
86 T: +30 210 6597331
87 F: +30 210 6597342
88 E-mail: smichal@bioacademy.gr

89
90 Sotirios Korossis, PhD
91 Centre for Biological Engineering, Wolfson School of Mechanical, Electrical and Manufacturing Engineering,
92 Loughborough University, Asby Road, LE11 3TU Loughborough, United Kingdom.
93 T: +44 1509 227651
94 Email: s.korossis@lboro.ac.uk

95

96 Introduction

97 Peripheral arterial disease is a highly prevalent and debilitating condition which affects more than 25 million patients in
98 Europe and North America alone (Criqui *et al.*, 1985; Fowkes *et al.*, 1991). Peripheral arterial disease leads to the
99 damage of blood vessels, which can be replaced by synthetic, autologous or allogeneic vascular grafts. Current
100 replacement approaches employ biocompatible materials solutions that are not able to regenerate or grow with the
101 patient. The gold standard for vascular grafting is autologous tissue, such as reversed saphenous vein graft (SVG)
102 (Koyama *et al.*, 2014). In despite of its low-cost and non-immunogenicity, autologous tissue does not represent the ideal
103 solution due to its limited availability and size mismatch (Kakisis *et al.*, 2005). Synthetic grafts, made of expanded-
104 polytetrafluoroethylene (ePTFE) or Dacron, have been reported to give successful outcomes in large diameter arterial
105 reconstructions (>8mm), but not for small diameter arterial reconstructions (< 5 mm) due to thrombosis and limited re-
106 endothelialization (Whittermore *et al.*, 1989; Yu *et al.*, 2013). In addition, cryopreserved arterial allogeneic grafts have a
107 limited availability and durability, due to calcification, immunogenicity and thrombogenicity (Daping *et al.*, 2009). The
108 shortcomings of the conventional grafts have prompt the investigation of new approaches for peripheral artery
109 reconstructions, such as the fabrication of tissue-engineered small-diameter vessels. Several studies have reported on
110 the development of small-diameter vascular using naturally derived proteins, such as collagen and fibrin (Clark *et al.*,

111 1995 and Swartz *et al.*, 2005, Dietrich *et al.*, 2012, Aper *et al.*, 2016, Schneider-Bartholda *et al.*, 2016), and synthetic
112 polymers (Roh *et al.*, 2010). However, the inadequate mechanical properties of these grafts (Chaouat *et al.*, 2006;
113 Zhang *et al.*, 2007; Zorlutuna *et al.*, 2009) have prompted workers in the field to adopt alternative approaches, such as
114 the use of decellularized tissue, which has been successfully used for tissue reconstructions, such as heart valves,
115 bladder, tendons and meniscus (Affonso da Costa *et al.*, 2005; Atala *et al.*, 2006; Ingram *et al.*, 2007; Bobylev *et al.*,
116 2014; Neumann *et al.*, 2014; Luo *et al.*, 2014; Sarikouch *et al.*, 2016,). Decellularization represents a promising
117 approach for overcoming the limited availability of autologous small-diameter vascular conduits, as well as the limited
118 availability of histocompatible allogeneic grafts since decellularisation has the potential to remove the major antigenic
119 determinants of allogeneic grafts. The latter has been effectively demonstrated by the successful translation of
120 decellularised aortic and pulmonary allogeneic heart valves in the clinical setting (Sarikouch *et al.*, 2016; Neumann *et al.*,
121 2014), whereas a number of studies have reported promising results with decellularized small-diameter vascular grafts
122 (Gui *et al.*, 2009, 2010; Hwang *et al.*, 2011; Dall'Olmo *et al.*, 2014;). The present study was a part of an overarching
123 project that was aimed at creating a small-caliber arterial graft for clinical use, utilizing the decellularised rat aorta. This
124 approach was chosen due to the arterial properties of the rodent graft and its suitable size for small-caliber arterial
125 reconstructions in humans. To the best of the authors' knowledge, such an approach has not been reported in the
126 literature. Dark Agouti (DA) rat abdominal aortas were decellularized using the widely employed detergent sodium-
127 dodecyl-sulfate (SDS) (Hashimoto *et al.*, 2010; Santoso *et al.*, 2014; Struecker *et al.*, 2014; Paniagua *et al.*, 2015) in 3-
128 [(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) buffer (Gui *et al.*, 2009, 2010; Hwang *et al.*, 2011;
129 Dall'Olmo *et al.*, 2014). Previously, we have reported on the effect of this modified decellularization protocol on the
130 mechanical and histological integrity of DA rat aortas *in vitro* (Katsimpoulas *et al.*, 2015). The present study was focused
131 on assessing the performance of the decellularised graft under orthotopic allogeneic transplantation in inbred Wistar (W)
132 rats without immunosuppression, prior to proceeding to heterotopic xenotransplantation. This specific animal model was
133 chosen since the inbred W and inbred DA rats differed in both their major and minor histocompatibility loci (MHC I and
134 II). Moreover, the orthotopic transplantation was chosen in order to assess the decellularised graft in the more
135 demanding, higher pressure environment of the aorta. The elicited immune response was evaluated in terms of
136 inflammatory cell infiltration into the implanted grafts, as well as in terms of the histoarchitectural and biomechanical
137 changes in the implanted grafts, after 6 weeks of implantation.

138 **Material and Methods**

139 **Experimental animals**

140 The study was conducted using 40 male inbred W and 20 male inbred DA rats, which were maintained in accordance
141 with the European Directive 2010/63 for the protection of animals used for scientific purposes and the "Guide for the
142 Care and Use of Laboratory Animals" (Guillen, 2012). All procedures were carried out in the animal facility of the
143 Biomedical Research Foundation of the Academy of Athens (EL25BIO003). The experimental protocol was approved by
144 the competent veterinary authority of the Biomedical Research Foundation of the Academy of Athens (7047/27-11-
145 2012), in accordance with the Greek legislation on the protection of experimental animals and the European Directive
146 2010/63.

147 ***Specimen procurement and dissection***

148 The abdominal aorta was approached via a midline laparotomy incision under isoflurane anaesthesia (IsoFlo, Abbott,
149 UK; 2.5%, 0.5 l/min O₂), as described by Onuta et al. (2007) and Hwang et al. (2011), and the side branches were
150 carefully cauterized (HTC, FLAB, Italy). The aorta was then mobilized and transected between a point distally to the left
151 renal artery and proximally to the iliac bifurcation. The harvested conduit was flushed with normal saline (Onuta et al.,
152 2007). Twenty native aortas from 10 DA (DA-W group; allogeneic control) and 10 W (W-W group; syngeneic control) rats
153 were orthotopically implanted untreated in W rats. Moreover, 10 DA aortas were decellularized using a modified protocol
154 (Katsimpoulas et al. 2015) and implanted orthotopically in W rats (n=10; decell DA-W group). The implanted aortas were
155 explanted after 6 weeks and tested histologically (n=2 from each group), immunohistochemically (n=2 from each group)
156 and biomechanically (n=6 from each group).

157 ***Decellularization***

158 The decellularisation of the rodent abdominal aortas was carried out as described previously (Gui et al., 2009) with
159 modifications (Katsimpoulas et al. 2015). Briefly, 20 aortas of approximately 15 mm in length were incubated with
160 CHAPS solution (8 mmol/L CHAPS [APPLICHEM, US], 1 mol/L NaCl, and 25 mmol/L EDTA in phosphate buffer saline
161 [PBS; Sigma, Germany]) at pH 8 for 22 h, followed by brief washes in PBS. The aortas were further incubated with SDS
162 solution (1.8mmol/L SDS [Sigma, Germany], 1mol/L NaCl, and 25 mmol/L EDTA in PBS) at pH7.5 for 24 h, followed by 3
163 washes, 5 min each, in PBS to completely remove the detergent. Finally, and modifying the previously described
164 protocol (Gui et al., 2010), the aortas were incubated at 37°C for 48 h in alpha minimal essential medium (a-MEM, Gibco
165 Life Technology, Germany), containing 40% (v/v) fetal bovine serum (FBS, Gibco Life Technology, Germany) and 1000
166 U/mL penicillin-streptomycin (Gibco Life Technology, Germany). FBS was added for removal of residual DNA from the
167 scaffolds (Gui L et al. 2010), since it contains DNAses and RNAses activated at 37°C in incubator. In addition, FBS can

168 be stored for a minimum 10 days at 4°C without altering its properties, while it has been approved by FDA for several
169 cellular and tissue engineered products. On the other hand, protocols that use external DNases and RNases or EGM-2
170 is of a higher cost due to the fact that a nuclease solution must be used immediately and is stable only for 3-5 h, creating
171 storage issues (Ingram, J.H., *et al.* 2007; Gui *et al.*, 2009). Moreover, this step does not alter the mechanical properties
172 of the scaffold, as assessed by our group (Katsimpoulas *et al.* 2015). All decellularisation steps were carried out under
173 agitation and sterile conditions.

174 ***Implantation procedure***

175 Anesthesia and aortic mobilization were performed as in the case of donor preparation while heparin was added in a
176 dose of 10 IU/100 g body weight intravenously. The aorta was transplanted just distally to the renal artery and proximally
177 to the iliac bifurcation in an end-to-end continuous fashion with 8-0 polypropylene suture (Prolene, Ethicon, USA). No
178 additional anticoagulation was administered postoperatively. Postoperatively, 5 mg/kg SC Carprofen (Rimadyl, Pfizer,
179 UK) daily and 150 mg/kg SC Amoxicillin (Amoxil, GlaxoSmithKline, UK) twice a day was administered for 3 days, for pain
180 and antibioprophylaxis, respectively. The recipient W rats were euthanized 6 weeks postoperatively, and the grafts, with
181 ~2cm surrounding tissue, were removed from the recipients.

182 ***Histology***

183 The tissue morphology and cellular content of the native, decellularised and explanted grafts was assessed by histology.
184 Whole aorta segments measuring 10 mm in length were isolated and fixed in 10% (v/v) neutral buffered formalin (Sigma-
185 Aldrich) overnight at 4°C. The samples were then dehydrated by sequential immersion in graded concentrations of
186 ethanol (70% v/v, 90% v/v and 100% v/v) in distilled water for 1h each, before they were immersed three times in xylene
187 for 1 h each time. The samples were then placed into metal molds that were partially filled with paraffin. The molds were
188 transferred onto a cold plate, to initiate wax solidification and secure sample orientation, and then were filled with
189 paraffin. The molds were placed onto the cold plate again, until the wax was completely solid. Subsequently, the paraffin
190 blocks were removed from the molds and sectioned using a microtome at a thickness of 6 µm. Circumferential sections
191 were cut from the central region of the grafts, in order to avoid the suture lines. Subsequently, the sections were
192 transferred into a water bath at 50°C and onto microscope slides. The slides with the tissue sections were dried on a
193 60°C hotplate. Prior to staining, the sections were dewaxed by sequential immersion in 2 individual pots with xylene for
194 10 min each, and were then sequentially rehydrated by immersion in graded concentrations of ethanol (2×5 min 100%
195 v/v, 1×2 min 95% v/v, 1×2 min 70% v/v) in distilled water. The general histoarchitecture of the grafts was evaluated using

196 standard haematoxylin and eosin (H&E; Merck) staining. Sections were immersed into Mayer's haematoxylin for 1 min,
197 rinsed under tap water for 5 min and then immersed into eosin for 3 min. Masson's trichrome was used to visualize
198 collagen fibre alignment and cell nuclei according to the method described by the manufacturer (Sigma-Aldrich). Briefly,
199 sections were treated with Weigert's iron haematoxylin for 5min, then in Biebrich scarlet-acid fuchsin and aniline blue for
200 6 and 5 min, respectively. Elastica Van Gieson was used to visualise elastic fibres and cell nuclei according to the
201 manufacturer's instructions (Sigma Aldrich). Briefly, sections were immersed in Weigert's resorcin fuchsin solution for 11
202 min, followed by Weigert's iron haematoxylin and picrofuchsin solution for 5 and 2 min, respectively. All stained sections
203 were dehydrated again in graded concentrations of ethanol (70% v/v, 90% v/v and 100% v/v) in distilled water, cleared
204 with xylene and mounted with Corbit Balsam mountant. Images were captured using a Nikon TE300 Eclipse light
205 microscope, incorporating a Nikon Digital Sight DS-U3 camera controller, and processed through the NIS-Elements D
206 Microscope Imaging Software (Nikon Instruments).

207 ***Immunohistochemistry***

208 The elicited immune response was evaluated in terms of inflammatory cell (T-cells, monocytes and macrophages)
209 infiltration by immunohistochemical staining. The presence of smooth muscle cells (SMCs)/fibroblasts and endothelial
210 cells (ECs) in the native and explanted grafts was also investigated by immunohistochemical staining. Whole aorta
211 samples measuring 10 mm in length, were isolated and fixed in zinc fixative at 4°C, dehydrated, and embedded in
212 paraffin wax, as described above. Circumferential sections (6 µm) were cut close to the central region of the samples,
213 dewaxed, and stained. Monoclonal antibodies against CD31 (ECs; PECAM-1(M-20)-R; Santa Cruz Biotech), calponin
214 (SMCs/myofibroblasts; 46794, Abcam), CD68 (monocytes and circulating and tissue macrophages; MCA341GA; AbD
215 Serotec), CCR7 (M1 macrophage sub-type; rabbit monoclonal Anti-CCR7 antibody [Y59], ab32527, Abcam) and CD206
216 (M2 macrophage sub-type; rabbit polyclonal to Mannose Receptor, ab64693, Abcam) were used to stain sections of
217 native and explanted aortas (Brown *et al.*, 2009; Brown *et al.*, 2012). Explanted aortas were also stained with anti-CD3
218 (T-cells at all stages of development; ab5690; Abcam) and anti-CD4 (T-regulatory and T-helper cells; ab125711; Abcam)
219 antibodies. Fresh porcine mitral valve posterior leaflet was used as positive control for calponin staining, whereas spleen
220 was used as positive control for T-cell staining. Isotype control antibodies (normal rabbit IgG for CD31, calponin and
221 CD3, Calbiochem; mouse IgG1 for CD4 and CD68, Dako) and omission of the primary antibody (secondary antibody
222 control) served as negative controls. Immunolabelling of anti-CD31, anti-CD68, anti-CD3 and anti-CD4 was carried out
223 using the EnVision®+ Dual Link System-HRP (DAB+) (K4065, Dako). Hydrogen peroxide (Sigma) was used to block
224 endogenous peroxidase. Images were captured under light microscopy, as described above. Immunofluorescence

225 staining was conducted for the immunolabelling of anti-calponin, anti-CCR7 and anti-CD206. Briefly, samples were
226 incubated in blocking medium (1% w/v BSA in PBS) for 60 min at RT, before they were washed once in TBS/Tween and
227 twice in TBS, for 5 min each time. Incubation in primary antibody was carried out for 1 h at RT. All samples were then
228 washed twice in TBS/Tween and twice in TBS for 5 min each and incubated with the secondary antibody (Alexa Fluor
229 488, donkey anti-rabbit, IgG, Jackson) for 30 min in the dark at RT. The samples were then washed shortly in
230 TBS/Tween and TBS and incubated in Roti®Mount FluorCare DAPI or in 1 μ M DAPI solution (Life Technologies) for 15
231 min at RT, followed by mounting. Images were captured using B-2A and UV-2A Nikon filters, with the microscope
232 described above.

233 **Biomechanical characterization**

234 The mechanical integrity of the explanted grafts was assessed under uniaxial tension. Longitudinal samples (n=6 for
235 each group) measuring 5mm in length by 2.5mm in width were isolated from the aortas and subjected to low strain-rate
236 uniaxial tensile loading to failure, according to the method reported previously (Korossis *et al.*, 2002), with minor
237 modifications. The testing was conducted in a Zwick/Roell tensile tester (model Z 0.5) equipped with a 200N load cell.
238 Prior to testing, the thickness of the samples was measured using a Sylvac position sensor equipped with a Kalibriert
239 force sensor (model: μ S246). The test sample was positioned between the position sensor and a bottom plate, and the
240 position sensor was lowered in a gradual and controllable manner, till it touched the sample and a force was registered.
241 The distance between the position sensor and bottom plate was recorded as the sample thickness. The thickness of the
242 samples was measured at three different points along their length, and averaged. Subsequently, the samples were
243 clamped at their ends, using sandpaper and a small amount of superglue to prevent slippage, under zero strain on the
244 tensile tester, which was set to produce a specimen preloading of 0.005 N before the operating program started to
245 acquire any data. During testing, the specimens were preconditioned for 10 cycles at a rate of 10 mm/min, before they
246 were sequentially stretched to failure at the same rate. Failure was assumed when the first decrease in load was
247 detected during specimen extension, whereas the mode of failure observed was near middle-section necking and
248 rupture for all the samples tested. The sample extension (Δl , in mm) and corresponding generated load (F, in Newtons)
249 that were recorded during the final loading to failure phase of the test, were converted to engineering strain (ϵ) and
250 engineering stress (σ , in MPa), respectively (Korossis *et al.*, 2002). The stress-strain behavior of each sample was
251 analyzed by means of six parameters (Korossis *et al.*, 2002), including the elastin (EI-E) and collagen (Col-E) phase

252 slopes, transition stress (σ_{Trans}) and strain (ϵ_{Trans}), ultimate tensile strength (σ_{UTS}) and failure strain (ϵ_{UTS}) . The
253 biomechanical parameters for each sample were calculated and averaged over the number of samples in each group.

254 **Data analysis**

255 The biomechanical testing results were presented as means together with their 95% confidence limits (C.I.). The data
256 was analyzed by one-way ANOVA, followed by calculation of the minimum significance difference (MSD). Statistical
257 significance was determined at the 0.05 confidence level.

258 **Results**

259 **Histological analysis**

260 The histological results of the native, decellularized and explanted syngeneic and allogeneic grafts are illustrated in
261 Figure 1 and at higher magnification in Figure 2. The results indicated that there were no apparent differences between
262 the histoarchitectures of native W and DA rats, with the aorta of both strains demonstrating a three-layered structure,
263 comprising the tunica intima (outer layer of cells facing the lumen), tunica media, consisting of SMCs, collagen fibers and
264 elastic fibers organized in a concentric fashion, and adventitia, mainly consisting of collagen fibers, fibroblasts and
265 loosely-aligned elastic lamellae (Callanan *et al.*, 2011; Allaire *et al.*, 2012; Katsimpoulas *et al.*, 2015). The decellularized
266 DA rat aorta was shown to be completely void of any observable cells and cellular debris, while it maintained the general
267 native trilaminar histoarchitecture with preserved collagen and elastic fibers. However, it also appeared swollen with
268 obvious gaps in the extracellular matrix (ECM), especially at the sites in the adventitial layer that were heavily populated
269 with cells.

270 The explanted syngeneic grafts (W-W; Figure 1 & 2) demonstrated an intact media with intact collagen and elastic fibers,
271 while their adventitia appeared more compact compared to their non-implanted control (W), suggesting the production of
272 ECM. The syngeneic grafts also presented a noticeable intimal thickening and cellular content throughout their
273 thickness. The explanted decellularized allografts (decell DA-W; Figure 1 & 2) showed a similar response to syngrafts,
274 with preserved elastin and collagen fibers in the media and a more compact adventitia compared to their non-implanted
275 control (decell DA). Moreover, they did not show any thickening of their intima, while they demonstrated a significant cell
276 repopulation throughout their thickness, as evidenced by the H&E staining. The latter observation highlighted the non-
277 cytotoxic nature of the decellularised grafts, which resulted in cell colonization and subsequent ECM remodeling. On the
278 other hand, the explanted allografts (DA-W; Figure 1 & 2) demonstrated disruption of the elastin and collagen network,
279 as well as cellular loss in the media, as evidenced by H&E and the distinct gaps in the sites that were previously

280 occupied by cells. Moreover, the DA-W allografts experienced cellular infiltration in the adventitia and adventitial fibrosis
281 that resulted in a significant thickening of their adventitia, as well as neointimal formation.

282 ***Immunohistochemical analysis***

283 The immunohistochemical results of the native and explanted syngeneic and allogeneic grafts are illustrated in Figure 3
284 (CD31), Figure 4 (CD68), Figure 5 (CD3 and CD4), Figure 6 (calponin), Figure 7 (CCR7) and Figure 8 (CD206). ECs
285 (CD31+) were observed in the lumen and vasa vasorum of the native DA and W aortas, and explanted syngrafts (W-W)
286 and untreated allografts (DA-W) (Figure 3). In contrast, no CD31+ cells were not visible on the explanted decellularized
287 allografts (decell DA-W; Figure 3). Putative SMCs or myofibroblasts (calponin+) were observed in the tunica media of the
288 native DA and W aortas, and the explanted syngrafts (W-W), but not in the tunica media of the explanted untreated (DA-
289 W) allografts (Figure 6). Instead, the untreated (DA-W) allografts demonstrated calponin+ cells only in their intima. No
290 calponin+ cells were observed in the decellularized (decell DA-W) allografts (Figure 6). The native DA and W aortas
291 were stained negative for CD68 (circulating and tissue macrophages and monocytes; Figure 4), whereas some CD68+
292 infiltrates were observed in the adventitia and intima of the explanted syngrafts (W-W; Figure 4). Extensive infiltration of
293 CD68+ cells was observed in the case of the untreated allografts (DA-W), which was spread uniformly throughout the
294 thickness of these grafts (Figure 4). CD68+ cells were also observed in the sub-endothelial region of the intima, as well
295 as the adventitia, of the explanted decellularized allografts (decell DA-W; Figure 4). However, the infiltration of CD68+
296 cells in the explanted decellularized allografts was only regional, and not spread throughout the graft, as in case of the
297 explanted untreated allografts. In spite of the lack for CD68+ staining in the native DA and W aortas, CCR7+ cells (M1
298 macrophage sub-type) were detected in these tissues (Figure 7). CCR7+ cells were also detected in the intima and
299 media of the explanted syngrafts (W-W; Figure 7). Similar observations were apparent in the case of the explanted
300 allografts (DA-W; Figure 7), in which CCR7+ infiltrates were observed in their intima and adventitia. On the other hand,
301 the explanted decellularized allografts (decell DA-W; Figure 7).demonstrated a milder infiltration of CCR7+ cells,
302 compared to the W-W and DA-W grafts, which was limited in their adventitia layer. No CD206+ cell infiltration was
303 detected in any of the in native tissues (DA and W), or syngraft (W-W), untreated allograft (DA-W) or decellularised
304 allograft (decell DA-W) explants (Figure 8).

305 No T-cell infiltration could be observed in the explanted syngrafts (W-W; Figure 5), whereas a significant infiltration of
306 CD3+ cells could be observed in the adventitia of the explanted untreated allografts (DA-W). Some localised CD3+ cell
307 presence could also be observed in the adventitia of the explanted decellularised allografts (decell DA-W; Figure 5), but

308 it was considerably reduced compared to the explanted untreated allografts (DA-W). The latter also demonstrated a
309 sporadic infiltration of CD4+ cells in their adventitia, in contrast to the explanted decellularised allografts (decell DA-W),
310 which did show any evidence of CD4+ cell infiltration (Figure 5). In general, the explanted untreated allografts (DA-W)
311 demonstrated a prominent infiltration of mononuclear cells, which was significantly reduced in the case of the explanted
312 syngrafts (W-W) and decellularised allografts (decell DA-W) (Figure 4 and 5). There was no unspecific staining observed
313 for both the secondary antibody and the isotype controls of all the above-mentioned antibody-stains (Figures 3, 4, 5, 6,
314 7, 8).

315 **Mechanical testing**

316 The mean biomechanical parameters of the explanted syngeneic and allogeneic grafts are illustrated in Figure 9. The
317 same figure also features the corresponding mean biomechanical parameters of native W (W) and DA (DA) rat aortas,
318 as well as of decellularised DA rat aortas (dec DA) that were produced using the same decellularisation protocol. This
319 data, which was obtained using the same uniaxial tensile testing protocol as in the present study, was adopted from
320 Katsimpoulas *et al.* (2015) and it was included in the analysis in order to better understand the potential changes in the
321 biomechanics of the scaffolds following implantation. Statistically significant differences were found only in the collagen
322 phase slope (Coll-E), ultimate tensile strength (σ_{UTS}) and thickness ($p=0.025$, 0.027 and 0.004 , respectively). In all three
323 cases, the statistically significant differences arose from the differences between the explanted untreated allograft (DA-
324 W) group and the other groups tested. Specifically, the DA-W group demonstrated the lowest Coll-E compared to the
325 other groups, with the difference being statistically significant only compared to the decellularized DA (decell DA) group
326 (MSD = 7.22). Similarly, the DA-W group achieved the lowest σ_{UTS} , with the difference being statistically significant when
327 compared to the W and decell DA groups (MSD = 2.07). Moreover, the average thickness of the DA-W group was higher
328 than any of the other groups, with the difference being statistically significant when compared to the native W and DA,
329 and decell DA groups (MSD = 0.11). The explanted decellularised allograft (decell DA-W) group showed no significant
330 differences compared to any of the other groups used in the study.

331 **Discussion**

332 The present study was a part of an overarching project that aimed at creating a small-caliber arterial graft for clinical use,
333 utilizing the decellularised rat aorta. Along these lines, the work was focused on assessing the performance of the
334 decellularised DA aorta, in terms of its biomechanical integrity and arterial compatibility, and potential immunogenicity,
335 under orthotopic allogeneic transplantation in W rats, prior to proceeding to heterotopic xenotransplantation in a large

336 animal in a future study. The orthotopic transplantation was chosen to assess the graft in the more demanding pressure
337 environment of the aorta. In this study, native and decellularised aortas from inbred DA rats were orthotopically
338 implanted into W inbred rats to simulate the allogeneic transplantation model, whereas W rat native aortas were
339 orthotopically implanted into W rats as syngeneic controls. To date, there has been a scarcity of studies on DA rat
340 tissues implanted into W rats; nevertheless, several studies have used tissues from other sub strains of W rats for
341 transplantation into DA rats (Mennander *et al.*, 1991; Mennander *et al.*, 1993). These rat strains differ in their major and
342 minor histocompatibility loci, and the immune response in allogeneic models with these strains, are mainly driven by the
343 MHC I and II present on donor cells (Schmitz-Rixen *et al.*, 1988). In this study, lymphocytes presence was investigated
344 using anti-CD3 and anti-CD4 antibodies, since CD3 has been reported to be a pan-T-cell marker (Aniansson Zdolsek *et*
345 *al.*, 1999) and CD4 a marker for T-regulatory and T-helper cells (Corthay *et al.*, 2009). Macrophages were detected by
346 the anti-CD68 antibody, which has been reported as a marker for pan-macrophages (Murray *et al.*, 2011), whereas the
347 M1 and M2 macrophage sub-types were detected by anti-CCR7 and anti-CD206 immunofluorescent antibodies,
348 respectively. M1 macrophages are typically activated by IFN- γ or lipopolysaccharides, producing proinflammatory
349 cytokines, and are indicative of an immune response, whereas M2 macrophages are activated by cytokines, such as IL-
350 4, IL-10, or IL-13, and produce either proliferation-inducing polyamines or proline to induce collagen production. M2
351 macrophages have been associated with wound healing and tissue repair and remodeling (Brown *et al.*, 2009; Valentin *et*
352 *al.*, 2009; Brown *et al.*, 2012; Jablonski *et al.*, 2015; Sager *et al.*, 2017)

353 Allograft transplantation usually leads to chronic rejection, which mainly consists of inflammation and intimal thickening
354 (Mennander *et al.*, 1992). In particular, this degenerative process induces a complete loss of SMCs in the media that, in
355 turn, induces media necrosis, elastin degradation, SMC migration towards the intima and intimal thickening throughout
356 the length of the graft (Mennander *et al.*, 1993). It has been hypothesized that medial necrosis might be due to a toxic
357 effect of inflammatory cells present in the adventitia, especially lymphocytes, on SMCs in the media (Mennander *et al.*,
358 1993). In the present study, media necrosis, partial elastic fiber degradation, SMC migration towards the intima and
359 lymphocyte (CD3+, CD4+) and macrophage (CD68+) infiltration in the adventitia were evidenced in the explanted
360 untreated allografts (DA-W), which also demonstrated a prominent increase in thickness of their adventitia (Figure 1 and
361 2). Donor ECs and SMCs were most probably the main antigenic targets of these infiltrates, since the DA-W grafts were
362 stained positively for CD31 in their lumen (Figure 3) and presented a significant content of calponin+ cells in their intima
363 (Figure 6). Similar results were described by Mennander *et al.* (1992) for DA rat thoracic aorta transplanted into Wistar

364 Furth rats. The authors reported a peak of inflammatory cells and increase in adventitial thickness after 2 months
365 implantation.

366 The syngrafts showed a mild immune response, with an intact media (Figure 2) and no lymphocyte presence (Figure 5).
367 Macrophages were observed in all explants, located mainly in the intima, media and adventitia of the allografts, and in
368 the media and intima of the decellularized allografts and syngrafts (Figure 4). In all explants, the macrophages were
369 identified as of the M1 sub-type (inflammatory; Figure 7), whereas no M2 macrophages (remodeling/repair) were
370 observed in any of the explants (Figure 8). In contrast to the DA-W allografts, the explanted decellularized allografts
371 (decell DA-W) induced an immune response similar to the syngrafts (W-W), with a reduced immune injury (Figure 2), low
372 amount of lymphocyte infiltrates (Figure 5), and macrophages that were localized mainly in regions of the adventitia
373 (Figure 7), although some macrophages were also observed in their intima region, as evidenced under CD68 staining
374 (Figure 4). This regional macrophage presence in the decell DA-W allografts might be due to inconsistencies in the
375 application of the decellularisation protocol, and subsequent cellular debris remnants in the grafts. However, these
376 results suggested that the decell DA-W allografts demonstrated a similar performance to their W-W syngrafts
377 counterparts, and that the decellularisation protocol was generally effective in removing the immunogenic material, such
378 as cellular debris, of the grafts.

379 The decellularized aortas, both prior (decell DA) and after (decell DA-W) transplantation, showed a well maintained
380 trilaminar structure, typical to abdominal rat aorta (Allaire *et al.*, 1994), with well-maintained collagen and elastic fibers
381 orientated concentrically along the circumferential direction in the media, and elastin lamellae in the adventitia (Figure 1
382 and 2). Elastic fibers and lamellae represent one of the main components of the aortic wall (Aikawa *et al.*, 2009;
383 Cavalcante *et al.*, 2011). Reduction in the number of elastic fibers and elastic lamellae has been reported to cause a
384 reduction of mural elastic resistance (Mello *et al.*, 2004) that could contribute to aneurysm formation (Boutouyrie *et al.*,
385 1992). The histological analysis of the decellularized aortas prior to implantation revealed absence of any observable
386 cells or cellular debris throughout the thickness of the arterial wall. The explanted decell DA-W allografts demonstrated
387 extensive cellular population in their media and adventitia, as well as evidence of remodeling of the adventitia, which
388 was rather disrupted by the decellularisation process (Figure 2). However, no ECs could be observed in the lumen of the
389 decell DA-W allografts, as evidenced by the CD31 staining (Figure 3), or any detectable SMC presence, as evidenced by
390 the calponin staining (Figure 5). On the other hand, the explanted DA-W allografts showed an overgrowth of their

391 adventitia, probably due to the recruitment of fibroblasts by inflammatory cells, in an attempt to replace the allogeneic
392 tissue (Mennander *et al.*, 1992), as well as neointima formation (Figure 2).

393 The histoarchitectural changes in the explanted DA-W allografts (Figure 1 and 2) had a direct effect on their mechanical
394 properties. Specifically, the DA-W group demonstrated on **average** the lowest Coll-E and σ_{UTS} , and the highest
395 macroscopic thickness, among **the** groups tested, indicating a deterioration of the integrity of these grafts **(Figure 9)**.
396 These changes in the mechanical properties of the DA-W allografts have the potential to induce long-term structural
397 failure. **Moreover, the reduced moduli measured for the DA-W explants, implied that there was a significant modulus**
398 **mismatch between these allografts and the native aorta of the recipients. This could have potentially generated**
399 **significant stress concentrations in the grafts and/or the surrounding aorta of the recipient, generating abnormal**
400 **biomechanical stimuli to infiltrating cells. In addition, the high stress concentration regions could have potentially**
401 **generated higher ECM damage, further attracting inflammatory and immune cells, and increasing the immunogenicity of**
402 **these grafts. On the other hand, and in the absence of a functional vasa vasorum, the increase in the DA-W graft**
403 **thickness *in vivo* could have generated progressively increased hypoxic conditions in the media of these grafts, which in**
404 **turn caused higher migration of immune cells, such as macrophages, macrophages, neutrophils, dendritic cells,**
405 **lymphocytes and immune lymphoid cells, whose characteristic ability is to infiltrate in tissues with low nutrients and**
406 **oxygen levels (Krzywinska *et al.*, 2018).**

407 In contrast, the explanted decell DA-W allografts showed no significant differences in the mechanical properties
408 compared to the native W rat aorta. Overall, these results suggested that the ECM of explanted decellularized scaffolds
409 was more stable and better maintained after 6 weeks implantation, in contrast to that of the untreated DA-W allografts,
410 which showed partial disruption of their elastic fibres, adventitia thickening and deteriorated mechanical properties,
411 caused by the immune response of recipients.

412 In conclusion, the results from this study demonstrated that the modified decellularisation protocol **used in the present**
413 **study** did not induce significant biomechanical or histological alterations of the DA rat aorta *in vivo*, whereas the immune
414 response of the recipients was improved by the decellularisation treatment compared to the untreated allografts. The
415 latter elicited a significant adverse immune response, which resulted in adventitia fibrosis and thickening, media necrosis
416 and neointima formation, whereas the syngrafts showed good tissue integration and mild immune response. **Future work**
417 **will assess the presence of nucleic acid and phospholipid remnants in the decellularised scaffolds. Moreover, future work**

418 will also assess the performance, remodeling and adaptation of the decellularised rat aorta graft in a heterotopic
419 peripheral artery porcine model.

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