**Completed checklist from ARRIVE guidelines 2.0. https://arriveguidelines.org/**

| | **Essential 10** | |
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| 1a | The groups being compared | The primary purpose was to evaluate the functional performance of low concentration SDS decellularised porcine pulmonary roots implanted into the right ventricular outflow tract (RVOT) of juvenile sheep over a 12-month period. The secondary objective was to explore cellular repopulation of the decellularised roots in vivo over time. This was achieved through monitoring of the animals using Doppler echocardiographic evaluation of the roots in situ and explantation of roots at 1, 3, 6 (n=4 per group for biological analysis) and 12 months (n=8; n=4 for biological analysis; n=4 for determination of material properties).<br><br>Ovine allografts implanted for 12 months (n=4) and non-implanted ovine pulmonary roots (n=4) were analysed for comparison of the longer term biological performance.<br><br>We did not include groups of ovine allografts for comparison of biological outcomes at 1, 3 and 6 months or material properties at 12 months since the performance of cardiac valve allografts in sheep was well documented in the literature and our previous studies of allogenic ovine aortic roots [31] in the RVOT of juvenile sheep had shown complete calcification (ie failure) after 6 months implantation. The material properties of the decellularised porcine pulmonary roots explanted at 12 months were compared with non-implanted ovine and non-implanted decellularised porcine roots. |
| 1b | The experimental unit | Single animal |
| 2a | Experimental units allocated to each group and total number in each experiment; total number of animals used | 26 juvenile (3 months old; male and female) Texel breed sheep were used in the study with a total of 22 animals implanted with decellularised porcine pulmonary roots and 4 animals implanted with ovine allografts. Two animals were lost during the peri-operative period (post-recovery from anaesthetic). One sheep suffered a cardiac arrest, and one sheep was lost due to pneumothorax. These sheep were replaced, hence a total of 26 sheep were included in the experimental study. |
| 2b | How sample size was decided | The study design enabled the in vivo functional performance of n=8 animals to be monitored over a 12-month period. This was based upon the larger sample size used in the literature in the field of implantation of decellularised cardiac roots in sheep which varied from n=3 to n=8. In order to minimise the use of animals, we selected a smaller sample size (n=4) at each time point for biological analysis of the cellular response because this secondary objective was an exploratory temporal study utilising a panel of 12 different antibodies to cellular markers and histochemical stains. |
| 3a | Inclusion or exclusion criteria and data points during the analysis | No criteria were set to exclude animals *a priori*. All animals were healthy at the start of the study. During the course of the study animals were excluded if they developed endocarditis (see 3b). |

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| 3b | Any animals not included in the analysis and why. | Two animals suffered endocarditis during the course of the study. Both of these animals were from the same group of animals. One died shortly before the 6-month time point and post-mortem analysis revealed endocarditis. The echocardiographic data for this animal was not available for analysis at the 6-month time point. A further root explanted at 6-months was found to have vegetations indicative of endocarditis. Upon histological analysis of a third root explanted at 6 months, there was evidence of microbial infection of the tissue. Thus, no further analyses (histology, immunohistochemistry) of any of the roots explanted at six months was undertaken. |
| 3c | Report exact value of n in each group | The exact value of n in each group for each analysis has been reported in Table (2), Table (3) and the Figure Legends for Figure (3), Figure (4) and supplementary Figure (1). |
| 4a | Randomisation of animals to control and treatment groups | Randomisation was not used since this was not a hypothesis driven controlled study |
| 4b | Strategy to minimise experimental confounders | Sheep were housed in covered open-air pens, to which they had be acclimatised, for the duration of the study. This was to minimise the risk of parasitic infection. |
| 5a | Blinding – who was aware of group allocation during allocation, conduct, outcome and data analysis | Sheep were allocated numerical identification numbers and Doppler Echocardiography was performed and evaluated by an independent specialist cardiologist, Dr Eduardo Balbi Mendel to avoid any observational bias.<br>To perform objective analysis, labelled slides were scanned on a Carl Zeiss Axio Imager.M2 incorporating an Axio Cam MRc5, which was controlled by Zen Pro software 2012 (Zeiss). Twelve fields of view (FoV; 100 x magnification) were identified in each section using a pre-determined template representing the adventitia, media and intimal regions of the distal, mid and proximal pulmonary artery wall, proximal and distal leaflet. The total number of cells in the DAPI stained sections and all the cells expressing a given marker within each FoV was counted using Image J software. This analysis was performed by a technician with no vested interest in the outcomes of the experiment.<br>For quantitative calcium analysis, samples were allocated a numerical identifier and sent to a third-party commercial laboratory for analysis. |
| 6a | Define outcome measures | Blood flow velocity across the valve, pressure gradient across the valve, leaflet function, calcification, internal diameter of roots. Histological evaluation of tissue structure, cellularisation by cell density and cell type, quantitative calcium content, material properties. |
| 7a | Statistical methods and software | Microsoft Excel 13 was used for descriptive statistics. Analysis of data from echocardiography (Table 2) and calcium content (Table 3) was performed in Excel 13 using one way analysis of variance. When significant variation was detected, the minimum significant difference between means was calculated using the T- |

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| | | method (Sokal RR and Rohlf FG Biometry 2nd ed. New York. WH Freeman & Company, 1980, p. 246).<br>Statistical tables (Rohlf F J and Sokal RR: Statistical Tables. New York, WH Freeman & Company, 1969) were used for arcsin transformations of percentage data. For analysis of cell density data and percentage cell density data which did not have equal variances, Welch ANOVA and Games-Howell pairwise comparisons to determine significant differences between means were performed in Minitab version 18.<br>For material properties, statistical analyses were performed using SPSS statistics software for Windows, Version 21.0 (Armonk, NY: IBM Corp.). One-way analysis of variance (ANOVA) was employed for evaluating the existence of significant variation among the valve groups. When significant variation was detected, Gabriel post hoc analysis was used to determine the significances between individual groups. |
| 8a | Species, strain, gender, age, weight | Texel breed sheep, male and female, 120 days old, 25.7 ± 2.7 Kg (mean ± 95% confidence limits) |
| 9a | Experimental procedures – what, where, when and why | Sheep were brought to the veterinary hospital 48 h before the surgical procedure, fasted and housed in a purpose built facility. Sheep were weighed and administered diazepam 0.5mg. $kg^{-1}$ and butorphenol 0.4mg. $kg^{-1}$ through an intravenous line and placed in lateral recumbency. Arterial pressure was monitored via direct arterial line in the radial artery. General anaesthesia was induced using Propofol at 4mg. $kg^{-1}$ and maintained using Propofol 0.5mg. $kg^{-1}$.min-1. Endotracheal intubation was performed and mechanical ventilation established. Arterial blood gases were monitored throughout. A left lateral thoracotomy was performed through the third intercostal space following Bupivacaine injection. The pericardium was opened and the heart and great vessels identified. Heparin 150 $U.kg^{-1}$ was given intravenously. Cardiopulmonary bypass was established through cannulation of the descending aorta and the right atrium, the pulmonary artery was dissected and clamped proximally and distally just before the bifurcation. A segment of the pulmonary artery was resected and the pulmonary valve leaflets were removed. The decellularised porcine or ovine allogeneic pulmonary root was then anastomosed using 5/0 prolene continuous suture proximally and distally. The clamps were removed and the function of the pulmonary root implant was observed.<br>Cardiopulmonary bypass was weaned ensuring that good oxygenation and pulmonary expansion were present. A drain was placed in the left pleural cavity and the wound was closed in three layers using Vicryl absorbable material. The chest drain was removed shortly after the confirmation of expansion of the lung by auscultation of the chest. The animals were allowed to surface from the anaesthesia, monitored for heart rate, respiratory rate and |

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| | | blood pressure continuously. A sample of arterial blood gases was processed to confirm adequate parameters to extubate. Animals were observed for their clinical behaviour, specially looking for any sign of discomfort and non-steroidal analgesia was administered intravenously if required. Once the anaesthetist had confirmed adequate postoperative status, the sheep was extubated, the arterial line removed and the sheep was then moved to a recovery room, where the animal continued under close observation for 2 h. Sheep were then housed in an outdoor grassed pen where water was freely available. The sheep were transported to the University farm "Fazenda Experimental Gralha Azul PUCPR" 24-48 h after the procedure. Sheep received prophylactic gentamicin 4mg.kg $^{-1}$ and cephalosporin 2mg kg $^{-1}$ until the 5th postoperative day.<br><br>The general health of the sheep was monitored by a veterinary surgeon throughout the in life-phase. The animals were weighed at the time of implantation and then at 1, 3, 6 and 12 months (for surviving animals). The animals were monitored by Doppler echocardiography at 1, 3, 6 and 12 months (for surviving animals). When warranted for humane reasons, euthanasia was conducted following veterinary advice, a full necroscopy was conducted to determine the cause of death. Implanted valves were re- retrieved by performing a redo left thoracotomy (as above) under general anaesthesia and the animals were sacrificed (KCl 19.1% iv) without recovery. The implanted pulmonary roots were retrieved. |
| 10a | Summary/descriptive stats for each experimental group plus variance | Summaries, descriptive statistics and 95% confidence intervals for all outcome measures are provided in:<br>Table (2): Quantitative data on sheep weights, internal valve diameter, blood flow velocity and mean pressure gradients across the roots in situ.<br>Table (3); Quantitative calcium content<br>Figure (3); Total number of cells in different tissue regions of the roots over time<br>Figure (4); Percentage cells expressing a given marker in different tissue regions of the roots over time<br>Supplementary Figure (1); Total number of cells expressing a given marker in different tissue regions over time. |

| | **Recommended set** | |
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| 11 | Abstract | The primary objective was to evaluate performance of low concentration SDS decellularised porcine pulmonary roots in the right ventricular outflow tract of juvenile sheep. Secondary objectives were to explore the cellular population of the roots over time. Animals were monitored by echocardiography and roots explanted at 1, 3, 6 (n=4) and 12 months (n=8) for gross analysis. Explanted roots were subject to histological, immunohistochemical and quantitative calcium analysis |

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| | | (n=4 at 1, 3 and 12 months) and determination of material properties (n=4; 12 months). Cryopreserved ovine pulmonary root allografts (n=4) implanted for 12 months and non-implanted cellular ovine roots were analysed for comparative purposes.<br><br>Decellularised porcine pulmonary roots functioned well and were in very good condition with soft, thin and pliable leaflets. Morphometric analysis showed cellular population by 1 month. However, by 12 months the total number of cells was less than 50% of the total cells in non-implanted native ovine roots. Repopulation of the decellularised porcine tissues with stromal ($\alpha$–SMA positive; vimentin positive) and progenitor cells (CD34 positive; CD271 positive) appeared to be orchestrated by macrophages (MAC 387 positive/ CD163 low and CD163 positive/ MAC 387 negative). The calcium content of the decellularised porcine pulmonary root tissues increased over the 12 month period but remained low (except suture points) at 401 ppm (wet weight) or below. The material properties of the decellularised porcine pulmonary root wall was unchanged compared to pre-implantation. There were some changes in the leaflets but importantly, the porcine tissues did not become stiffer.<br><br>The decellularised porcine pulmonary roots showed good functional performance in vivo and were repopulated with ovine cells of the appropriate phenotype in a process orchestrated by M2 macrophages, highlighting the importance of these cells in the constructive tissue remodelling of cardiac root tissues. |
| 12a | Background, rationale and experimental approach | The introduction provides a full background to the clinical need (paragraph 1), a review of studies of the use of decellularised allografts (paragraph 2), a review of previous clinical studies of decellularised porcine valves (paragraph 3), our previous studies in the field (paragraph 4), the rationale for the study (paragraph 5) and the objectives (paragraph 6) together with a justification for the choice of animal model. |
| 12b | Relevance to humans | This is stated at the end of the introduction: “The juvenile sheep was selected as the model for the study due to similar valve biomechanics and haemodynamics to the human. The juvenile sheep implant model is used in cardiac valve research because it is an excellent predictor of the durability and performance of biologic heart valves as affected by calcification. |
| 13 | Objectives | The primary objective of this study was therefore to evaluate the functional performance of decellularised porcine pulmonary roots implanted into the right ventricular outflow tract over juvenile sheep over a 12-month period. |

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| | | The secondary objective was to explore cellular repopulation of the decellularised roots in vivo over time. |
| 14 | Ethical statement | The *in vivo* study was conducted at the University Veterinary Hospital "Hospital Veterinário Para Animais De Companhia" PUC-PR São José Dos Pinhais, Brazil. The study was performed in accordance with the institutional guidelines for animal care and were approved by the Ethical Committee of Researches PUC-PR (project number CEUA 511). |
| 15 | Housing and husbandry | Sheep were brought to the veterinary hospital 48 h before the surgical procedure, fasted and housed in a purpose-built facility. Numbered ear tags were used to aid individual identification. . Sheep were weighed and administered diazepam 0.5mg. $kg^{-1}$ and butorphenol 0.4mg. $kg^{-1}$ through an intravenous line prior to the operative procedure, which is described in Section 2.6 "Surgical procedure and animal husbandry". Following the procedure sheep were moved to a recovery room, where the animal continued under close observation for 2 h. Sheep were then housed in an outdoor grassed pen where water was freely available. The sheep were transported to the University farm "Fazenda Experimental Gralha Azul PUCPR" 24-48 h after the procedure. Sheep received prophylactic gentamicin 4mg.$kg^{-1}$ and cephalosporin 2mg $kg^{-1}$ until the 5th postoperative day. |
| 16 | Animal care and monitoring | The general health of the sheep was monitored by a veterinary surgeon throughout the in life-phase. The animals were weighed at the time of implantation and then at 1, 3, 6 and 12 months (for surviving animals). The animals were monitored by Doppler echocardiography at 1, 3, 6 and 12 months (for surviving animals). |
| 17 | Interpretation/scientific implications | The interpretation and scientific implications of the study have been thoroughly discussed in relation to the literature in the Discussion (Section 4). The conclusions were "The low concentration SDS decellularised porcine pulmonary roots showed good functional performance in the RVOT of sheep over a 12-month period. The decellularised pulmonary root tissues were repopulated with ovine cells of the appropriate phenotype in a process orchestrated by macrophages of an M2 phenotype, highlighting that these cells may be important in the constructive tissue remodelling of cardiac root tissues. Following 12 months in vivo, however, the extent of cellular repopulation was less than 50% of the cellular population in native ovine valved conduit tissues of a similar age. Longer term studies would be required to determine whether the stromal cell densities reached those of native sheep pulmonary valve tissues. Importantly, the fact that the leaflets, the primary location of valve dysfunction, were populated |

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| | | with stromal cells, indicated the potential for matrix repair and remodelling".<br>The limitations of the study have been discussed in the second to last paragraph of the discussion. "There are several limitations to this study. The low sample size at each time point, limited the statistical power, and there was a high level of biological variation within the groups. The loss of histological and immunohistochemical data at the 6-month time point was a limitation but this did not impact the outcome of the study. Although an extensive number of antibodies were used in the immunohistochemical analysis, this could have been extended to additional markers. The native ovine pulmonary roots from 15-month old sheep used for comparison in the biomechanical and morphometric analyses were significantly smaller than the 12- month explanted decellularised cryopreserved porcine pulmonary roots ". |
| 18 | Generalisability/translation | The results indicated that the low concentration SDS decellularised porcine pulmonary roots functioned well in the RVOT of sheep over a 12 month period and show the potential for future clinical translation.<br>The results also add important insight into the role of macrophages in the constructive remodelling response to implanted biological scaffolds in vivo. |
| 19 | protocol registration | NA |
| 20 | Data Access | All data related to this study is freely available in the Leeds Data Repository University of Leeds. [Dataset].<br>Tayyebeh Vafaee, Fiona Campbell, Dan Thomas, João Gabriel Roderjan, Sergio Veiga Lopes, Francisco DA da Costa, Amisha Desai, Paul Rooney, Louise M Jennings, Helen Berry, John Fisher and Eileen Ingham (2022): Implantation of decellularized porcine pulmonary heart valves in sheep - dataset. [Dataset]. https://doi.org/10.5518/1102 |
| 21 | Declaration of interests | Statements have been added at the end of the manuscript:<br>Acknowledgements:<br>This research was funded through WELMEC, a Centre of Excellence in Medical Engineering funded by the Wellcome Trust and EPSRC, under grant number WT 088908/Z/09/Z and through the Medical Technologies Innovation and Knowledge Centre (phase 2 - Regenerative Devices), funded by the EPSRC under grant number EP/N00941X/1 as Proof of Concept award PoC015 "In vivo evaluation of acellular porcine pulmonary roots". The study was partially supported by Tissue Regenix Group PLc. We would like to thank Ms Nicola Conway (Research Technician) for histology and immunohistochemistry support.<br><br>Conflicts of interest statement: |

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| | | At the commencement of this work, Helen Berry was an employee of Tissue Regenix Group Plc. The study was partially supported by Tissue Regenix Group Plc. Helen Berry, Eileen Ingham and John Fisher are shareholders in Tissue Regenix Group Plc. FDA da Costa has a financial relationship with Tissue Regenix Group PLc at the time of the study. |