

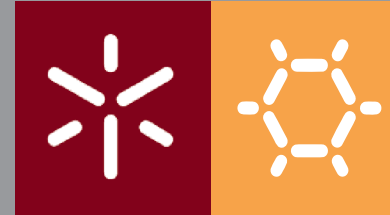


Laura Passos Morgado Franco Frazão

**The Decellularized Human Chorion
Membrane as a New Biomaterial:
characterization and applications**

Universidade do Minho

I3Bs - Instituto de Investigação em Biomateriais, Biodegradáveis e Biomiméticos





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Membrane as a New Biomaterial:
characterization and applications**

Tese de Doutoramento em Engenharia de Tecidos,
Medicina Regenerativa e Células Estaminais

Trabalho efetuado sob a orientação do
Professor Nuno João Meleiro Alves das Neves

Junho de 2021

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

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The decellularized human chorion membrane as a new biomaterial: characterization and applications

ABSTRACT

Decellularized inner body membranes have been studied for several different tissue engineering applications. However, its availability is limited, except in the case of placental membranes. Placental membranes are composed by the amnion membrane and the chorion membrane. While the human amnion membrane has been widely studied for tissue engineering and regenerative medicine applications, the human chorion membrane was poorly studied, only being subject of research interest in recent years.

Envisioning the potential of decellularized human chorion membrane (dHCM) to be used in tissue engineering applications, a decellularization protocol was developed for the human chorion membrane. The dHCM was characterized regarding its composition and mechanical properties. Additionally, the dHCM was used as a substrate to support cell cultures for the creation of a blood brain barrier (BBB) *in vitro* model, as a surgical patch for the creation of a vascular graft and also for the treatment of congenital diaphragmatic hernias.

dHCM showed cytocompatibility with human and mice endothelial cells (EA.hy296, HUVECs, and b.End3), human fibroblasts (MRC5), and mice astrocytes (C8-D1A). The angiogenic properties of the dHCM were also investigated. Moreover, dHCM demonstrated hemocompatibility and antibacterial activity against *S. aureus*. After *in vivo* implantation, dHCM integrated well in the host tissue after 28 days when implanted subcutaneously in mice. Nevertheless, when sutured into a pig diaphragm, dHCM was fully integrated by the host tissue after 3 weeks.

Altogether, the works developed in this thesis, allowed for a detailed characterization of dHCM for the first time. It was demonstrated that dHCM has unique characteristics that make it a valuable new biomaterial to be use as substrate to support cell cultures and as a surgical patch.

Keywords: Chorion membrane; Decellularization; Blood brain barrier; Vascular graft; Diaphragmatic hernia; Biomaterial; Surgical patch

A membrana do córion humano descelularizada como um novo biomaterial: caracterização e aplicações

RESUMO

As membranas de órgãos internos descelularizadas têm sido estudadas para diferentes aplicações em engenharia de tecidos. No entanto, a disponibilidade destas membranas é limitada, com exceção das membranas da placenta. A placenta é composta por duas membranas, a membrana amniótica e a membrana do córion. Enquanto a membrana amniótica humana descelularizada foi extensivamente estudada para aplicações em engenharia de tecidos e medicina regenerativa, a membrana do córion só recentemente despertou interesse na comunidade científica para estas aplicações.

Para estudar o potencial da membrana do córion humano descelularizada (dHCM) em engenharia de tecidos, foi otimizado um protocolo de descelularização eficiente para esta membrana. A dHCM foi caracterizada em relação à sua composição e propriedades mecânicas. Posteriormente, a dHCM foi utilizada como substrato de suporte ao crescimento de células para a criação de um modelo *in vitro* da barreira hemato-encefálica, como um enxerto cirúrgico para a criação de enxertos vasculares e também para o tratamento de hérnias diafragmáticas.

A dHCM mostrou ser citocompatível com células endoteliais de humano e ratinho (EA.hy296; HUVECs, e b.End3), fibroblastos humanos (MRC5) e astrócitos de ratinho (C8-D1A). As propriedades angiogênicas da dHCM foram investigadas e foi demonstrado que a dHCM é compatível com o sangue humano e tem atividade antimicrobiana contra *S. aureus*. Após implantação *in vivo*, a dHCM começou a ser integrada pelo tecido hospedeiro após 28 dias de implantação subcutânea em ratinhos. No entanto, quando suturada ao diafragma de porcos, a dHCM mostrou capacidade de integração no tecido hospedeiro após 3 semanas.

Os trabalhos desenvolvidos nesta tese permitiram obter uma caracterização detalhada da dHCM, previamente inexistente na literatura. Adicionalmente, foi demonstrado que a dHCM tem características únicas, tornando-a um novo biomaterial promissor como substrato de suporte ao desenvolvimento de culturas de células e como enxerto cirúrgico.

Palavras-chave: Membrana de córion; Descelularização; Barreira hemato-encefálica; enxerto vascular; hérnia diafragmática; biomaterial; enxerto cirúrgico

TABLE OF CONTENTS

ACKNOWLEDGMENTS III

STATEMENT OF INTEGRITY IV

ABSTRACT V

RESUMO VI

TABLE OF CONTENTS VII

LIST OF ABBREVIATIONS..... XIV

LIST OF FIGURES XVII

LIST OF SUPPLEMENTARY FIGURES XXI

LIST OF TABLES..... XXII

SHORT *CURRICULUM VITAE* XXIII

LIST OF PUBLICATIONS XXIV

INTRODUCTION TO THE THESIS FORMAT XXVI

SECTION 1 1

GENERAL INTRODUCTION..... 1

CHAPTER I - THE APPLICATION OF DECELLULARIZED INNER BODY MEMBRANES IN TISSUE
ENGINEERING: CURRENT STATUS AND FUTURE PERSPECTIVES· 3

Abstract 3

I-1. Introduction 4

I-2. Decellularized Inner Body Membranes..... 4

I-2.1. Connective Tissue Membranes..... 7

I-2.2. Epithelial Membranes 9

I-2.3. Placental Membranes 11

I-3. The Availability of Inner Body Membranes..... 12

I-4. Decellularized Inner Body Membranes in Tissue Engineering 13

I-4.1. Cardiovascular applications..... 18

I-4.2. Musculoskeletal applications 23

I-4.3.	Skin applications.....	28
I-4.4.	Fetal membranes applications	30
I-4.5.	Ocular applications	31
I-4.6.	In vitro models.....	32
I-4.7.	Other applications.....	33
I-5.	Final Remarks.....	34
I-6.	Funding	35
I-7.	References.....	35
SECTION 2.....		51
EXPERIMENTAL DESIGN		51
CHAPTER II - MATERIALS AND METHODS		53
Overview		53
II-1.	Materials	54
II-1.1.	Human chorion membrane (HCM)	54
II-1.2.	Polycaprolactone (PCL)	54
II-2.	Methods	55
II-2.1.	Production Techniques	55
II-2.2.	Decellularized human chorion membrane (dHCM) characterization.....	56
II-2.3.	In vitro assays.....	60
II-2.4.	<i>In vivo</i> assays	71
II-2.5.	Microbiology	75
II-3.	Statistical Analysis	75
II-4.	References	76
SECTION 3.....		79
DHCM OBTENTION AND CHARACTERIZATION.....		79
CHAPTER III		80
CHAPTER III - METHOD TO DECELLULARIZE THE HUMAN CHORION MEMBRANE.....		81
Abstract		81
III-1.	Introduction	82
III-2.	Methodology	83

III-2.1. Overview	83
III-2.2. Materials	84
III-2.3. Solutions	84
III-2.4. Equipment	84
III-2.5. Separate the chorion membrane from human placenta	85
III-2.6. Decellularization protocol	86
III-2.7. DNA content measurement	87
III-3. Pitfalls and limitations	88
III-4. Discussion	89
III-5. Conclusions	91
III-6. Funding	91
III-7. Acknowledgements	91
III-8. References	91
CHAPTER IV - DECELLULARIZED HUMAN CHORION MEMBRANE AS A NOVEL BIOMATERIAL FOR TISSUE REGENERATION **	97
Abstract	97
IV-1. Introduction	98
IV-2. Materials and Methods	99
IV-2.1. Human Placentas	99
IV-2.2. Decellularization of human chorion membrane (HCM)	99
IV-2.3. DNA extraction and quantification	100
IV-2.4. Histological analysis	100
IV-2.5. Scanning electron microscopy (SEM)	100
IV-2.6. Swelling assay	101
IV-2.7. Collagen quantification	101
IV-2.8. Sulfated glycosaminoglycans quantification	101
IV-2.9. Protein extraction and quantification	101
IV-2.10. SDS-PAGE	102
IV-2.11. Dot-Blot	102
IV-2.12. Immunolocalization of proteins	102
IV-2.13. Mechanical properties	103
IV-2.14. Cytotoxicity analysis	103

IV-2.15.	<i>In vivo</i> studies	103
IV-2.16.	Statistical analysis	104
IV-3.	Results	105
IV-3.1.	HCM decellularization and ECM protein retention.....	105
IV-3.2.	dHCM's mechanical properties.....	108
IV-3.3.	Biocompatibility of dHCM <i>in vitro</i> and <i>in vivo</i>	109
IV-4.	Discussion	111
IV-5.	Conclusions	114
IV-6.	Funding.....	115
IV-7.	Acknowledgments.....	115
IV-8.	References	115
IV-9.	Supplementary material	120
SECTION 4.....		121
DECELLULARIZED HUMAN CHORION MEMBRANE (dHCM) APPLICATIONS.....		121
CHAPTER V - DECELLULARIZED HUMAN CHORION MEMBRANE AS A SUBSTRATE TO CREATE A BBB <i>IN VITRO</i> MODEL		123
Abstract		123
V-1.	Introduction	124
V-2.	Materials and Methods.....	125
V-2.1.	Decellularized human chorion membrane (dHCM).....	125
V-2.2.	Cell culture and metabolic activity	125
V-2.3.	Fluorescence imaging.....	126
V-2.4.	TEER measurements	126
V-2.5.	Permeability assays	126
V-2.6.	Transferrin uptake assay.....	127
V-2.7.	Immunofluorescence	128
V-2.8.	Statistical analysis	128
V-3.	Results	128
V-3.1.	dHCM trophoblast side is more suitable for a BBB <i>in vitro</i> model.....	128
V-3.2.	BBB model validation.....	132
V-3.3.	Functional BBB permeability	133

V-4.	Discussion	136
V-5.	Conclusions	137
V-6.	Funding	137
V-7.	Acknowledgments	138
V-8.	References	138
CHAPTER VI - THE DECELLULARIZED HUMAN CHORION MEMBRANE AS A VASCULAR GRAFT		
143		
Abstract		143
VI-1.	Introduction	144
VI-2.	Materials and Methods.....	145
VI-2.1.	Decellularized human chorion membrane (dHCM)	145
VI-2.2.	Tube formation assay and reendothelization assay	145
VI-2.3.	Quantification of angiopoietin 2.....	146
VI-2.4.	Chick chorioallantoic membrane (CAM) assay.....	146
VI-2.5.	Platelet adhesion assay	146
VI-2.6.	Measurement of platelet adhesion using lactate dehydrogenase (LDH) assay.....	147
VI-2.7.	Hemolytic activity assay.....	147
VI-2.8.	Protein adsorption	148
VI-2.9.	Microbiology.....	148
VI-2.10.	dHCM rolling.....	149
VI-2.11.	dHCM tube resistance to peristaltic movements.....	149
VI-2.12.	Mechanical properties	149
VI-2.13.	Scanning electron microscopy (SEM)	149
VI-2.14.	Fluorescence imaging and Immunocytochemistry	150
VI-2.15.	Statistical analysis	150
VI-3.	Results	150
VI-3.1.	dHCM biocompatibility and recruitment of endothelial cells <i>ex ovo</i>	150
VI-3.2.	dHCM thrombotic potential.....	153
VI-3.3.	dHCM hemolytic potential	154
VI-3.4.	dHCM antibacterial properties	155
VI-3.5.	Tubular construct and mechanical properties.....	157
VI-4.	Discussion	158

VI-5.	Conclusions	162
VI-6.	Funding	162
VI-7.	Acknowledgements	162
VI-8.	References	162
CHAPTER VII		168
CHAPTER VII - NOVEL DIAPHRAGMATIC HERNIA PORCINE MODEL TO STUDY THE INFLUENCE OF SUBJECT GROWTH: A COMPARISON STUDY BETWEEN GORE-TEX		169
Abstract		169
VII-1.	Introduction	170
VII-2.	Materials and Methods.....	171
VII-2.1.	Patch preparation.....	172
VII-2.2.	Animal care and use.....	173
VII-2.3.	Chest x-rays	173
VII-2.4.	Anesthesia and analgesia	173
VII-2.5.	Surgical technique.....	174
VII-2.6.	Post-operative care	174
VII-2.7.	Euthanasia and necropsy evaluation	175
VII-2.8.	Outcome measurements	175
VII-2.9.	Histology.....	176
VII-2.10.	Immunohistochemistry.....	176
VII-2.11.	Statistically analysis.....	176
VII-3.	Results	177
VII-3.1.	dHCM is associated with higher surgical time	177
VII-3.2.	PCLem and dHCM are associated with hernia recurrence	178
VII-3.3.	PCLem and dHCM are associated with better tissue integration.....	180
VII-4.	Discussion	183
VII-5.	Conclusions	187
VII-6.	Funding.....	187
VII-7.	Acknowledgements	187
VII-8.	References	187
VII-9.	Supplementary Material	192

SECTION 6..... 193

GENERAL CONCLUSIONS..... 193

CHAPTER VIII 194

CHAPTER VIII - GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES..... 195

 VIII-1. General conclusions..... 195

 VIII-2. Future Perspectives 196

LIST OF ABBREVIATIONS

#

°C – Degrees Celsius
 µL – Microliter
 µm – Micrometer
 µg – Microgram
 % - Percentage
 3D – 3-Dimensional

A

α-MEM – Alpha-minimum essential medium
 Abs – Absorbance
 ANG2 – Angiopoietin 2
 ANOVA – Analysis of variance
 ATCC – American type culture collection

B

BBB – Blood brain barrier
 b.End3 – Mouse brain capillary endothelial cell line
 bFGF – Basic fibroblast growth factor
 BSA – Bovine serum albumin
 BM – Basement membrane
 BMP – Bone morphogenetic protein
 bp – Base pairs

C

CaCl₂ – Calcium chloride
 CAM – Chorioallantoic membrane
 CDH – Congenital diaphragmatic hernia
 CDHSG – Congenital diaphragmatic hernia study group
 CFU – Colony forming units
 cm - Centimeter
 cm² – Centimeter square
 CNS – Central nervous system
 COL I – Collagen type I
 CO₂ – Carbon dioxide
 CTR – Control
 C8-D1A – Mouse cerebellum astrocyte cell line

D

DAB – 3, 3 -diaminobenzidine
 DAPI – 4,6-diamidino-2-phenylindole
 DGAV – Portuguese general directorate of food and veterinary
 dHAM – Decellularized human amniotic membrane
 dHCM – Decellularized human chorion membrane
 DMB – Dimethylmethylene blue

DMEM – Dulbecco's modified Eagle's medium
 DNA – Deoxyribonucleic acid
 DNase – Deoxyribonuclease
 D-PBS – Dulbecco's Phosphate-Buffered Saline
 dsDNA – Double stranded DNA

E

EA.hy926 – Human umbilical vein cell line
 ECM – Extracellular matrix
 EDC – 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
 EDTA – Ethylenediamine tetraacetic acid
 EGF – Epidermal growth factor
 ELISA – Enzyme-linked immunosorbent assay
 EMT – Epithelial to mesenchymal transition
 ERDF – European regional development fund
 EU – European Union

F

FBS – Fetal bovine serum
 FCT – Portuguese foundation for science and technology
 FDA – U.S. Food and Drug Administration
 FITC – Fluorescein Isothiocyanate
 FGF – Fibroblast growth factor

G

g – Grams
g – Times gravity
 GAG – Glycosaminoglycan
 GFP – Green fluorescence protein
 GLUT – Glucose transporter

H

h – Hour
 HA – Hyaluronic acid
 HAM – Human amniotic membrane
 HCl – Hydrochloric acid
 HCM – Human chorion membrane
 hCMEC/D3 – Human cerebral endothelial cell line
 HIV – Human immunodeficiency virus
 HMD – Hexamethyldisilane
 HUVECs – Human umbilical cord vein endothelial cells
 H&E – Hematoxylin-eosin

I

IM – Intramuscular
 IPPROM – Iatrogenic preterm premature rupture of membranes
 iPSC – Induced pluripotent stem cell
 ISO – International organization for standardization
 IV – Intravenous

K

kDa – Kilodalton
 kg – Kilogram
 kN – Kilonewton
 kV – Kilovolt

L

LDH – Lactate dehydrogenase
 LESC – Limbal epithelial stem cell
 LSCD – Limbal epithelial stem cell deficiency

M

mg – Milligram
 MgCl₂ – Magnesium chloride
 min – Minute
 mL – Milliliter
 mm – Millimeter
 mM – Millimolar
 mmHg – Millimeter of mercury
 MMP – Matrix metalloproteinase
 MPa – Megapascal
 MRC-5 – Human fetal lung fibroblast cell line
 MT – Masson's trichrome
 MTT/MTS – Tetrazolium
 Mw – Molecular weight

N

n – Number of samples
 NaCl – Sodium chloride
 NADH – Nicotinamide adenine dinucleotide
 NADPH – Nicotinamide adenine dinucleotide phosphate
 NaOH – Sodium hydroxide
 NFM – Nanofibrous mesh
 ng – Nanograms
 nm – Nanometer
 NSAID – Non-steroidal anti-inflammatory
 NVU – Neurovascular unit

P

p – Statistical level of significance

PATH – Doctoral Program on Advanced Therapies for Health

PBS – Phosphate-buffered saline
 PCL – Polycaprolactone
 PCLem – Polycaprolactone electrospun meshes
 PET – Polyethylene terephthalate
 pg – Picogram
 PGG – Penta-galloyl glucose
 PLA – Poly(lactic acid)
 PLACL – Poly(L-lactic acid)-co-poly(e-caprolactone)
 PLGA – Polylactic-co-glycolic acid
 PPP – Platelet-poor plasma
 PR – Patch repair
 PRP – Platelet-rich plasma
 PTFE – Polytetrafluorethylene

R

RBC – Red blood cells
 rpm – Rotations per minute
 RT – Room temperature

S

s – seconds
S. aureus – *Staphylococcus aureus*
 SDS-PAGE – Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
 SCID – Severe combined immunodeficient
 SD – Standard deviation
 SDS – Sodium Dodecyl Sulfate
 SEM – Scanning electron microscopy
 SIS – Porcine small intestinal submucosa
 SMA – Smooth muscle actin
 S1P – Sphingosine 1-phosphate

T

TBS – Tris buffered saline
 TEER – Transendothelial electrical resistance
 Tie – Tyrosine-protein kinase
 TL – Trophoblast layer
 TNF- α – Tumor necrosis factor alpha
 TGF- β – Transforming growth factor beta
 TRITC – Tetramethylrhodamine
 TSB – Tryptic soy broth media

U

UV – Ultraviolet light

V

v – Volume

VEGF – Vascular endothelial growth factor

LIST OF FIGURES

Figure I-1 Representation of human inner body membranes which have been used in decellularization-related studies, divided in three groups 1) Epithelial membranes (pericardium, pleura, and peritoneum that includes mesentery and omentum); 2) Connective tissue membranes (fascia that includes periosteum, and synovial membrane); 3) Placental membranes (amniotic and chorion membranes). 6

Figure III-1 The human chorion membrane (HCM). (A) Scheme and **(B)** Hematoxylin/Eosin staining of the native HCM in a transversal cut. The different layers of the HCM – reticular layer, basement membrane, and trophoblast layer - are identified in the figure..... 83

Figure III-2 Separation of the HCM from the human placenta 85

Figure III-3 Scheme of the HCM decellularization protocol. 87

Figure III-4 dHCM characterization. (A-B) Transversal sections of DAPI staining in native HCM **(A)** and dHCM **(B)**. **(C)** SEM micrograph of dHCM in a transversal cut. **(D)** Content of double-stranded DNA in native HCM and dHCM. **(E)** Electrophoresis in agarose gel of DNA extracted from both native HCM and dHCM. 88

Figure IV-1 Human chorion membrane decellularization. Representative transversal sections of Hematoxylin/Eosin (H&E) staining of HCM **(A)** and dHCM **(B)**. Representative SEM micrographs of dHCM from the reticular layer side **(C)** and the trophoblast side **(D)**. Representative transversal sections of DAPI staining from HCM **(E)** and dHCM **(F)**. Double-stranded DNA (dsDNA) quantification in HCM and dHCM **(G)**. Agarose gel electrophoresis of DNA extracted from HCM and dHCM **(H)**. Thickness of air-dried HCM and dHCM measured with a picometer in, at least, three different sites **(I)**. Top view of HCM **(J)** and dHCM **(K)** cut into pieces of 2 cm x 2 cm. Swelling behavior of dHCM in culture medium and D-PBS **(L)**. Where, ** $p \leq 0.01$ and *** $p \leq 0.001$ 106

Figure IV-2 dHCM protein composition. Representative lanes of two different samples from an SDS-PAGE gel of the digested HCM and dHCM **(A)**. Representative dot blot results for collagen type I, collagen type IV, fibronectin and laminin, in both native and dHCM tissues. Digestion buffer without samples was used as a negative control, while the native protein was used as a positive control **(B)**. Immunolocalization of collagen type I, collagen type IV, fibronectin and laminin in representative transversal sections of HCM and dHCM **(C)**. Quantification of soluble collagen **(D)**, insoluble

collagen (E) , and sulfated glycosaminoglycans (GAGs) (F) content in HCM, dHCM, and TL in $\mu\text{g}/\text{mg}$ of dry tissue. Where $** p \leq 0.01$; $*** p \leq 0.001$ and $**** p \leq 0.0001$	108
Figure IV-3 Mechanical properties of native and decellularized HCM. Stress-strain curves of HCM (A) and dHCM (B) . Ultimate tensile strength (C) and Young's modulus (D) of both HCM and dHCM. Where $**** p \leq 0.0001$	109
Figure IV-4 Mechanical properties of native and decellularized HCM. EA.hy926 (A) and MRC-5 (B) cells' metabolic activity in commercial inserts (CTR), dHCM trophoblast layer side and dHCM reticular layer side. Representative images of H&E and Masson's trichrome staining of dHCM subcutaneously implanted on balb/c mice in three different time-points (4, 10 and 28 days) (C) . Where, $* p \leq 0.05$; $*** p \leq 0.001$ and; $**** p \leq 0.0001$	111
Figure V-1 Schematic representation of bEnd3 and C8-D1A cells seeding on both sides of dHCM in mono and co-culture conditions	129
Figure V-2 The suitability of dHCM for a BBB <i>in vitro</i> model. Metabolic activity (A - B) and phalloidin/DAPI staining (C - D) of bEnd.3 (A and C) and C8-D1A (B and D) cells in dHCM trophoblast side and dHCM reticular layer side in both mono and co-culture conditions at days 3 and 7 of culture.	131
Figure V-3 BBB model validation. TEER values were obtained for bEnd.3 monolayer (A) and for the system that is composed by bEnd.3 monolayer and dHCM (B) , both in mono and co-culture conditions. Diffusion assays with and 70 kDa FITC-dextran (C) were also performed for mono and co-cultures, at two different time-points (days 3 and 7). Where $** p \leq 0.01$	132
Figure V-4 Functional BBB permeability. Different molecules were used to exemplify the different transport systems at the BBB. Caffeine was used for membrane diffusion (A) , glucose for carrier-mediated transport (B) , and transferrin for receptor-mediated transport (C) . Where $* p \leq 0.05$; $** p \leq 0.01$	134
Figure V-5 GLUT-1 and transferrin receptor expression. GLUT1 (A) and transferrin receptor (B) expression were assessed over time (days 3 and 7) in both conditions (mono and co-culture) by immunofluorescence.	135
Figure VI-1 Endothelial cell compatibility. Phalloidin staining was used to assess the ability of HUVECs to form capillary-like structures (marked with a white triangle) in CTR (Matrigel) condition, dHCM reticular layer side and dHCM trophoblast layer side at 4, 8 and 12 h (A) . For the same	

conditions at the same time-points, angiopoietin 2 (ANG 2) production was quantified (B) . A reendothelization assay was also performed in three different conditions (CTR (coverslip), dHCM reticular layer side, and dHCM trophoblast layer side). After 48 h HUVECs were stained with phalloidin (red) and for Von Willebrand Factor (green) Where, * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$	151
Figure VI-2 CAM Assay. Representative photographs of the chorionic and allantoic epithelium of <i>ex ovo</i> CAM after 7 days from implantation in control (CTR) condition (without material) and in both dHCM sides: reticular layer and trophoblast layer (A) . Several parameters were quantified in all conditions such as number of blood vessels (B) , vascular density (C) , and vascular length density (D)	152
Figure VI-3 dHCM thrombotic potential. Plasma protein adsorption was quantified in CTR condition (glass surface) and in both sides of dHCM (A) . Platelet adhesion was analyzed for the same conditions using isolated platelets (B) and platelet poor plasma (PPP) (C) . Isolated platelets on the three different surfaces were stained with phalloidin (D₁-D₃) . Moreover, SEM micrographs were obtained to verify platelet morphology (D₄-D₆) . Where, *** $p \leq 0.001$, **** $p \leq 0.0001$	153
Figure VI-4 dHCM hemolytic potential. The hemolytic ratio was obtained for the CTR (glass surface) condition and for both sides of dHCM. For the assay positive and negative control, 1% SDS and PBS were added to the RBC suspension, respectively (A) . SEM micrographs of RBC seeded into the different surfaces were obtained (B) . Where **** $p \leq 0.0001$	155
Figure VI-5 dHCM resistance to infection. dHCM antibacterial activity against <i>S. aureus</i> adherence and attachment, photographs of contact plates in CTR (polypropylene films) (A₁) , dHCM reticular layer side (A₂) and dHCM trophoblast layer side (A₃) . CFU quantification in all surfaces (B) . Where, ** $p \leq 0.01$ and *** $p \leq 0.001$	156
Figure VI-6 dHCM tubular structure. dHCM was rolled creating a tube with 3-4 mm diameter (A) and variable length (B) . The walls of the dHCM tube were characterized regarding its thickness (C) . Representative SEM micrographs of a transversal cut of the dHCM tube (D) , of the inner side of the dHCM tube, the reticular layer side (E) , and of the outer side the dHCM tube, the dHCM trophoblast layer side (F)	157
Figure VI-7 Mechanical characterization of the dHCM tube. dHCM tube was mounted in 3D CulturePro™ Bioreactor attached to a peristaltic pump and submitted to a flow rate of 42.7 mL/min	

(A-B). Longitudinal and circumferential Young's modulus (C) and ultimate tensile strength (D) of dHCM were determined. Despite bending the tubular graft, the flow is not blocked (E-H).....	158
Figure VII-1 Experimental timeline. Farm male pigs were acclimatized for 7 days and then submitted to a chest X-ray and thoracoscopic surgery for patch implantation. Seven weeks post-surgery, animals were submitted to a second chest X-ray, thoracoscopy, laparoscopy and were euthanized.	172
Figure VII-2 Thoracoscopic defect and repair of the diaphragm. Representation of trocars site (A). Thoracoscopic creation of a diaphragm defect (B). Repair of the diaphragm defect with Gore-Tex® (C), PCLem (D) and dHCM (E-H).....	178
Figure VII-3 Outcome Measurements. Descriptive table summarizing different outcome measures, such as surgery duration (min), follow up time (days), recurrence rate (%), weight gain from surgery to euthanasia (kg), and adhesion formation grade at time of death per animal (A). Representative chest x-ray image priori to (B) and post-surgery, at time of euthanasia, in Sham (C) Gore-Tex® (D), and PCLem (E) groups. Since all animals of dHCM group were submitted to humane endpoints, no chest X-rays were performed post-surgery.	180
Figure VII-4 Macroscopic and microscopic evaluation of new diaphragmatic tissue. Top view of Gore-Tex® (A), PCLem (B) and dHCM (C) after follow-up time. Comparison of inflammation, angiogenesis and fibrosis mean scores (score 0-3) between groups, defined by the histological analysis of H&E and MT staining. Two different tissue areas were analyzed, lesion site that corresponds to the patches and transition that represents the native diaphragm at the site of the sutures (D).	182
Figure VII-5 Immunohistochemistry for α-SMA, CD31 and CD105. Representative sections of α -SMA, CD31 and CD105 staining of the native diaphragm, in both tendon (A, I and O, respectively) and muscle (B, J and R, respectively). Patch samples were divided into two distinct zones: lesion and transition (Figure VII-2C, VII-2D and VII-2H). Representative sections of α -SMA, CD31 and CD105 for Gore-Tex® (C-D, K-L, S-T, respectively), PCLem (E-F, M-N, U-V, respectively) and dHCM (G-H, O-P, W-X, respectively) groups.	183

LIST OF SUPPLEMENTARY FIGURES

Supplementary Figure IV-1 Representative images of H&E staining of control animals used in the subcutaneous assay performed on BALB/c mice at three different time-points (4, 10 and 28 days). In sham animals, only the surgery was performed (without dHCM implantation). Empty pockets were performed on the contralateral side of animals implanted with dHCM.120

Supplementary Figure VII-1 Sham animals’ characterization. Thoracoscopic sham surgery without defect creation **(A)**. Representative sections of α -SMA **(B)**, CD31 **(C)** and CD105 **(D)** staining of diaphragm section from sham animals.....192

LIST OF TABLES

Table I-1 Summary of the clinical tissue engineering applications of the decellularized inner body
membranes..... 16

Table VII-1 Scoring system175

SHORT *CURRICULUM VITAE*

Laura Passos Morgado Franco Frazão was born on the 3rd of September 1992 in Lisbon, Portugal. She is currently a PhD student at 3B's Research Group, I3Bs Research Institute on Biomaterials, Biodegradables and Biomimetics, at University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine at Avepark at Caldas das Taipas, Guimarães, Portugal.

Laura has graduated in Health Sciences at the University of Lisbon, Portugal with a final grade of 14/20, in 2013. During that time, she did a summer internship in Instituto de Tecnologia Química e Biológica António Xavier (ITQB), at Nova University of Lisbon, Portugal.

She proceeded to a master's degree in Neurosciences at the University of Lisbon, Portugal, which was successfully concluded in 2016, with a final grade of 18/20. The dissertation studies were developed in Portuguese Institute of Oncology of Lisbon where she worked in the genetic profiling of pediatric brain gliomas, which resulted in a publication.

Later in 2016, she started her PhD at the 3B's Research Group, University of Minho, Portugal, under the supervision of Prof. Dr. Nuno Neves in the field of ECM-based biomaterials.

Scientifically, Laura has contributed with 2 peer-reviewed papers in international journals (plus 2 submitted), 2 book chapters, and an international patent application (WO/2021/005531). Furthermore, she participated in national and international conferences with 1 oral communication and 4 poster presentations.

LIST OF PUBLICATIONS

The work performed during the PhD period resulted in the publications listed below:

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Frazão, L. P., Neves, N.M. Decellularized Inner Body Membranes: tissue engineering and clinical applications (submitted).

Book Chapters

Frazão, L. P.; Vieira de Castro, J.; Nogueira-Silva, C.; Neves, N.M. (2020) Method to decellularized the human chorion membrane. In: Caballero D.; Kundu S.; Reis R. (eds). *Methods in Cell Biology*, vol 157. Academic Press, Elsevier.

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Oral communications

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Frazão, L. P.; Neves, N.M. “Mimicking the Basement Membrane in a Blood-Brain Barrier In Vitro Model”, TERM STEM, Porto, Portugal (2017-11-13 to 2017-11-17).

Frazão, L. P.; Neves, N.M. “An In-depth Study of the blood-brain barrier structure and function”, Chem2Nature 2nd School, Porto, Portugal (2017-06-05 to 2017-06-09).

Patents

International patent application WO/2021/00553 *Decellularized human chorion membrane as a new substrate to mimic biological barriers, method, kit and uses thereof*. **Frazão, L.P.**, Vieira de Castro, J. Reis, R.L., Neves, N.M.

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INTRODUCTION TO THE THESIS FORMAT

The work developed under the scope of this PhD is organized into five **Sections (1 to 5)**, comprising eight **chapters (I to VIII)**. This structure allows for a logical and thematic sequence of the presented and discussed topics.

Section 1 comprises a general introduction to the use of decellularized inner body membranes in tissue engineering (**Chapter I**). It is followed by **Section 2** where a detailed description of the Materials and Methods used in this thesis is provided (**Chapter II**). In **Section 3**, it is described the method to decellularize the human chorion membrane (**Chapter III**) and the decellularized human chorion membrane (dHCM) is characterized (**Chapter IV**). **Section 4** comprises the applications of dHCM in different tissue engineering areas, such as the creation of a blood-brain barrier *in vitro* model (**Chapter V**), the creation of a vascular graft (**Chapter VI**), and its use as a surgical patch for diaphragmatic hernia repair (**Chapter VII**). Since these works are based in a series of publications, the chapters are presented as an adapted version of the published or submitted manuscripts, maintaining its contents (abstract, introduction, experimental section, results, discussion, conclusion, and references). The publication is identified in the front page of each chapter. Finally, on **Section 5 (Chapter VIII)**, the overall conclusions of the work developed in this thesis are described as well as its future perspectives.

Section 1 – General Introduction

Chapter I – The Application of Decellularized Inner Body Membranes in Tissue Engineering: Current Status and Future Perspectives: This chapter provides an overview on the application of decellularized inner body membranes in tissue engineering. Firstly, inner body membranes are identified and characterized, and then, they are analyzed for cardiovascular, musculoskeletal, skin, fetal membranes, and *in vitro* models' applications. Final remarks on the availability of inner body membranes and its use in the clinic are also presented.

Section 2 – Experimental Design

Chapter II – Materials and Methods: In this chapter, it is provided a detailed description of the materials and production techniques used. Moreover, the used methodologies for dHCM characterization are described. The *in vitro* and *in vivo* procedures applied to validate biologically the potential of the membrane described on section 3 and 4 are also identified and explained.

Section 3 – Decellularized human chorion membrane obtention and characterization

Chapter III – Method to decellularize the human chorion membrane: This chapter describes, for the first time, the decellularization protocol used to successfully decellularize the human chorion membrane, resulting in a compact and easy to handle membrane. This chapter is based on the international patent application WO/2021/00553 *Decellularized human chorion membrane as a new substrate to mimic biological barriers, method, kit and uses thereof*. Nevertheless, instead of the patent, the adaptation of the associated publication comprises this chapter to ensure the clarity of the thesis.

Chapter IV – Decellularized human chorion membrane as a novel biomaterial for tissue engineering: dHCM is characterized for the presence and distribution of cell nuclei, DNA, and extracellular matrix (ECM) components. dHCM mechanical properties and its *in vitro* and *in vivo* biocompatibility are also studied.

Section 4 – Decellularized human chorion membrane applications

Chapter V – A functional blood-brain barrier *in vitro* model using decellularized human chorion membrane: A functional BBB *in vitro* model was created by seeding brain capillary endothelial (b.End3) cells into dHCM in mono or co-culture with astrocytes (C8-D1A). The model was characterized by transendothelial resistance (TEER) and paracellular permeability using 4 kDa and 70 kDa FITC-dextran. Moreover, the functionality of the model was studied by using different molecules associated with different transportation mechanisms across the BBB (membrane transport, carrier-mediated transport, and receptor-mediated transport).

Chapter VI – The decellularized human chorion membrane as a vascular graft: The dHCM was used as a substrate to produce small diameter vascular grafts. Endothelial cell cytocompatibility and dHCM angiogenic properties were investigated, as well as dHCM hemocompatibility and antibacterial activity. Moreover, dHCM was rolled onto a tube and its resistance to a peristaltic flow was analyzed.

Chapter VII – Novel biodegradable membranes compared with Gore-Tex® for the treatment of congenital diaphragmatic hernia: A growing large animal model (*Sus scrofa domesticus*) was used to compare dHCM with polycaprolactone electrospun mesh (PCLem) and the clinical gold standard (Gore-Tex®) for the treatment of diaphragmatic hernias using thoracoscopic techniques. The outcomes measured were surgery duration, animal's weight gain, adhesions, grade, and recurrence rate. Regarding graft integration in the host tissue, inflammation, angiogenesis, and fibrosis grades were quantified. Moreover, specific markers for muscle and vessel formation were identified.

Section 5 – General conclusions

Chapter VIII – General conclusions and future perspectives: In this chapter, the general conclusions of the works presented in the previous sections are reported. Moreover, the limitations and potential benefits of the work developed in this thesis are discussed, as well as the future perspectives and research lines that may follow this work.

SECTION 1

GENERAL INTRODUCTION

Chapter I

The Application of Decellularized Inner Body Membranes in Tissue Engineering: Current Status and Future Perspectives

Chapter I

The Application of Decellularized Inner Body Membranes in Tissue Engineering: Current Status and Future Perspectives

ABSTRACT

The main objective of tissue engineering and regenerative medicine is to create functional biological substitutes able to maintain, improve or renew the function of a whole organ or a tissue. For that, it is important to mimic the natural environment of cells, the extracellular matrix (ECM). Decellularization is a technique that allows the maintenance of the native ECM while removing its cellular components. So, the use of decellularized matrices in tissue engineering applications is associated with inductive cell invasion, and promotion of remodeling processes including vascularization and innervation. Moreover, they are well tolerated by xenogeneic recipients. Inner body membranes are thin sheets composed by cells and ECM that cover the surface of internal organs and lines body cavities. Decellularized inner body membranes have been explored to create biocompatible and functional tissue constructs for several tissue engineering applications such as cardiovascular, musculoskeletal, and ocular diseases, skin wounds, fetal membrane disorders and even in *in vitro* models. Nevertheless, there are no standardized decellularization or post-decellularization processing protocols for inner body membranes, limiting their broad use in clinics. In the present review, all these aspects will be explored.

*This chapter is based on the following publication:

Frazão L. P.; Neves N. M., (2020), "The Application of Decellularized Inner Body Membranes in Tissue Engineering: Current Status and Future Perspectives" (submitted).

I-1. INTRODUCTION

The main objective of tissue engineering and regenerative medicine is to use methods of life sciences and engineering to create functional biological substitutes able to maintain, improve or renew the function of a whole organ or a tissue [1–5]. It comprises the involvement of cells, signaling molecules and scaffolds that support and rely upon each other [1]. Scaffolds may be defined as constructs that mimic the natural structure surrounding the cells, the extracellular matrix (ECM). Scaffolds may be obtained from synthetic, natural materials or a combination of both [6]. When compared with synthetic, biological scaffolds are associated with a higher biocompatibility, lower cytotoxicity and inflammatory reaction [7,8].

There is a variety of processing methods available to obtain biological scaffolds, such as electrospinning, or 3D printing [1,9]. Nevertheless, given the complexity and incomplete understanding of ECM composition and structure, it is difficult to create an ECM scaffold that fully mimics the native ECM [4]. Decellularization is an approach that allows the maintenance of the native ECM while removing its cellular components. So, decellularized matrices offer additional advantages such as the induction of cell invasion, giving cues for cell modulation and behavior and the promotion of remodeling processes that will allow the formation of a vascularized, innervated, and functional tissue [3,10–13].

Inner body membranes are sheets of cells and ECM that cover the surface of internal organs and line body cavities. Decellularized inner body membranes are commonly used to prepare biocompatible and functional tissue constructs [14].

The aim of this review is to explore the use of decellularized inner body membranes in tissue engineering. Thus, inner body membranes will be characterized and divided in three main groups (epithelial membranes, connective tissue membranes, and placental membranes). Then, inner body membranes will be analyzed for cardiovascular, musculoskeletal, skin, fetal membranes and *in vitro* models' applications.

I-2. DECELLULARIZED INNER BODY MEMBRANES

The main goal of decellularization is to remove all cells and genetic material from the extracellular matrix (ECM), removing potential antigens that can cause an inflammatory response or immune-mediated implant rejection [4,15]. It was established that to avoid host and cell adverse effects, decellularized

tissues should lack detectable amounts of nuclear material (stained with DAPI), its DNA fragments should have less than 200bp and the amount of double-stranded DNA/mg of dry tissue should be less than 50 ng [16]. Moreover, decellularization aims to maintain the structural, biochemical and biomechanical properties of the ECM [4].

There are several decellularization approaches using mechanical, chemical and enzymatic techniques and/or its combinations. Mechanical procedures comprise agitation, sonication, mechanical pressure, or freeze-drying. They are normally used to disrupt the cell membrane and are usually combined with chemical approaches, generally washing steps using ionic solutions or detergents. For enzymatic techniques, trypsin, dispase, esterases or nucleases are the most used enzymes [9,17]. An extended review of all the decellularized protocols used in decellularized inner body membranes can be found in [18].

Inner body membranes are divided into two main types, connective tissue membranes and epithelial membranes [19]. Since pregnancy is a temporary state, another group of inner body membranes was included, the placental membranes. In this section, inner body membranes will be described regarding its composition and function. Inner body membranes are represented in Figure I-1.

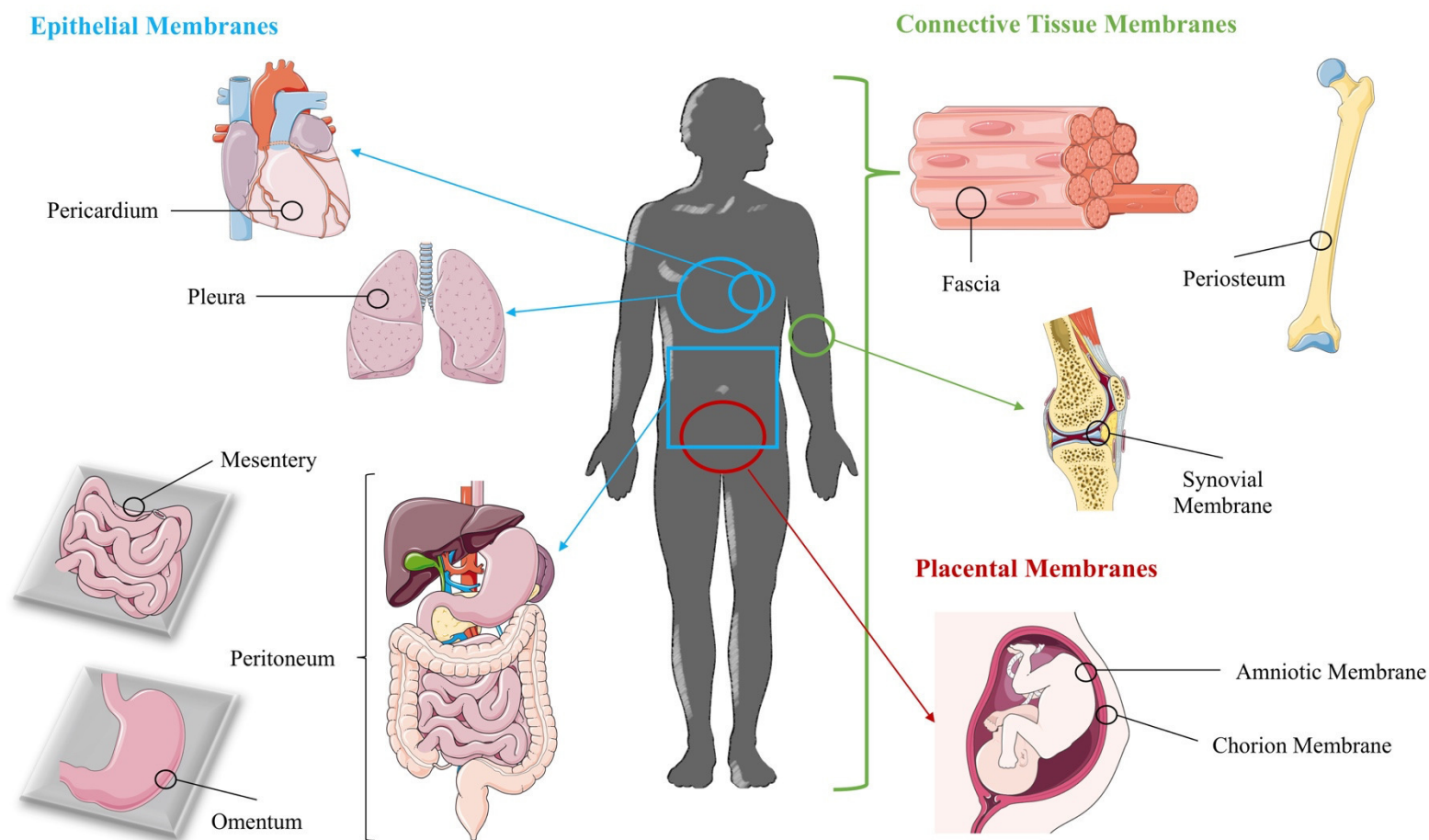


Figure I-1 Representation of human inner body membranes which have been used in decellularization-related studies, divided in three groups 1) Epithelial membranes (pericardium, pleura, and peritoneum that includes mesentery and omentum); 2) Connective tissue membranes (fascia that includes periosteum, and synovial membrane); 3) Placental membranes (amniotic and chorion membranes).

I-2.1. Connective Tissue Membranes

Connective tissue membranes are composed by various types of connective tissues and encapsulate organs [19]. Connective tissue membranes include fascia, periosteum and synovial membrane [14,19], that will be described below.

I-2.1.1. Fascia

Despite the scientific uncertainty regarding “fascia” definition, there is agreement that fascia is a connective tissue membrane that surrounds and holds every organ, blood vessel, bone, nerve fibers, and muscle in place. The fascial tissue has an ubiquitous distribution in the body, creating various layers at different depths and forming a three-dimensional metabolic and mechanical matrix [20]. Four different fascial planes can be distinguished: the meningeal fascia, the visceral fascia, the superficial fascia, and the axial or deep fascia. The meningeal fascia surrounds the central nervous system [21,22]. The visceral fascia extends from the cranial base to the pelvic cavity, covering all organs. This fascia guides the neurovascular and lymphatic packets towards the organs [22]. The superficial fascia or pannicular fascia is the lowermost layer of the skin and it blends with the reticular dermis [22]. The axial fascia, investing fascia or deep fascia is a dense fibrous connective tissue that surrounds muscles, bones, nerves, and blood vessels. This fascia has a high content of collagen fibers and has elastin fibers giving it strength, integrity and extensibility and resilience. The deep fascia can have different nomenclature according with its location, when it encloses the nerves (apineurium, perineurium and endoneurium), the muscles (epimysium, perimysium and endomysium), the blood vessels (tunica externa), the cartilage (perichondrium), or the bones (periosteum, endosteum) [22]. The periosteum will be also discussed below.

There are some studies concerning the decellularized fascia. To the best of our knowledge, all of them focus on the deep fascia that surrounds muscles or bones. Excluding the periosteum, most studies focus on the decellularization of human fascia lata (the fascia that surrounds the thigh) [23,24], with two commercial products being available, the Tutoplast® and Suspend [25,26]. There is also a study on the decellularization of porcine anterior rectus fascia (on the rectus abdominal muscles) [27].

I-2.1.2. Periosteum

The periosteum is a dense connective tissue that adheres to bone surfaces [28]. Anatomically, the periosteum covers the majority of the bony structures with the exception of their intra-articular surfaces and sesamoid bones [29]. Moreover, periosteum is a vital functional structure for bone formation and remodeling [30,31], having a major role in bone growth and repair and an impact on the blood supply of bone and skeletal muscle [29]. Periosteum is structurally divided into two different layers: the outer fibrous layer and the inner layer (or cambium layer) [32]. The fibrous outer layer is primarily composed by collagen and is poor in cells [33]. This layer is subdivided into two parts, the superficial and the deep portions. The superficial part of the outer layer is the most highly vascularized substratum of the periosteum, significantly contributing to the blood supply of bone and skeletal muscle. Moreover, it also presents a rich neural network. On the other hand, the deep portion of the outer layer is fibroelastic and not highly vascularized [29]. In contrast, the inner layer of the periosteum is highly cellular and composed by mesenchymal progenitor cells, differentiated osteogenic progenitor cells, osteoblasts and fibroblasts in a thin collagenous matrix. The thickness of the cambium layer decreases with age, being undistinguishable from the outer layer in adulthood. Consequently, the osteoblastic potential of the periosteum also decreases with age [29]. Some evidences suggest that the osteoblastic potential of periosteum is also influenced by location, showing less osteogenic potential in calvarial periosteum when compared with tibia periosteum [32,34].

As an alternative to periosteal autografts, associated with a limited amount of available healthy periosteum, decellularized periosteum from rabbits have been studied [31,33,35].

I-2.1.3. Synovial Membrane

The synovial membrane lines the spaces of diarthrodial joints, tendon sheaths and bursae. It comprises a continuous surface layer of cells (intima) and an underlying tissue (subintima). The intima layer is composed by synoviocytes (fibroblast-like cells) and macrophages embedded in the ECM. The subintima layer contains fibroblasts and lymphatic and blood vessels in a collagenous ECM. Between the intimal surfaces is a small amount of fluid rich in hyaluronic acid, providing cartilage lubrication and a stable non-adherent surface. Other functions of the synovial membrane are arranging the volume of synovial fluid and control the nutrition and composition of chondrocytes in the joints [36].

Decellularized synovial membranes were already obtained from the femorotibial and femoropatellar joints of healthy horses as an alternative to cartilage ECM [37,38].

I-2.2. Epithelial Membranes

Epithelial membranes are composed by a layer of epithelial tissue and a layer of fibrous connective tissue [19]. Epithelial membranes represent the mesothelium that comprises the pericardium, peritoneum (that includes mesentery and omentum), and pleura [14,19], that will be described below.

I-2.2.1. Pericardium

The pericardium or pericardial sac is a membrane that surrounds the heart. Its main functions are to hold the heart in place, to prevent overfilling of the heart, to provide lubrication and to function as a physiological barrier, isolating the heart from other organs and avoiding spread of infections. The pericardium is composed by two layers, the inner layer (serous pericardium) and the outer layer (fibrous pericardium) that enclose the pericardial cavity, that contains a lubrication fluid, minimizing the friction when the heart contracts. The serous pericardium is further divided into two layers, the internal visceral layer that forms the outer layer of the heart (epicardium) and the outer parietal layer that lines the internal surface of the fibrous pericardium (continuous with the central tendon of the diaphragm). It is a rigid structure, composed by tough connective tissue that prevents rapid overfilling of the heart [39,40].

There are several studies involving decellularized pericardium from mice [41], humans [42,43], pigs [44–50], and horses [51]. Nevertheless, bovine pericardium is the most studied [52–68], with two commercial products available Bioripar® and Tutomesh®.

I-2.2.2. Peritoneum

The peritoneum is the largest and the most complex membrane in the body. It covers the inner lining of the abdominal cavity and supports the internal organs within. The peritoneum is a continuous membrane composed by a basement membrane that contains mesothelial cells and a thin connective tissue carrying blood vessels, nerves, fat, lymphatic ducts, and fibers. It has two layers and a potential space between them (peritoneal cavity). The outer layer, the parietal peritoneum, lines the abdominal and the pelvic walls, while the inner layer, visceral peritoneum, covers the internal organs. The peritoneal

cavity consists in mesothelial cells that are responsible for the production of a small amount of serous fluid that allows the two peritoneum layers to slide freely over each other [69]. The peritoneum is a highly folded structure and there are several different terms to describe the folds and spaces that are part of the peritoneum. In this work, we will focus on the most relevant decellularized inner body membranes. So, mesentery and omentum will be described below.

There are several studies exploring the decellularized peritoneum, normally harvested from pigs [70–74].

I-2.2.2.1 Mesentery

The mesentery is a double layer of peritoneum caused by invagination of an organ into the peritoneum. It connects the intraperitoneal organ to the posterior abdominal wall, serving as a conduit for blood vessels, nerves and lymphatic ducts going to and from the organ in question. Moreover, the mesentery is associated with lipid and glycemic metabolism through the production of C-reactive protein. The mesentery from the small intestine is called “mesentery” and prevents the collapse of intestines towards the pelvis when the body posture is standing upright. Nevertheless, mesentery related to other parts of the gastrointestinal system is named according to the viscera it connects to [75].

A limited number of studies were performed using decellularized mesentery from mice [76] and pig [77,78].

I-2.2.2.2 Omentum

The omentum is structurally similar to the mesentery since it results of abdominal structures formed from the peritoneum. It is composed by sheets of visceral peritoneum that extend from the stomach and the proximal part of the duodenum to other abdominal organs. The omentum is divided in the greater omentum and in the lesser omentum. The greater omentum consists of 4 layers of visceral peritoneum that descend from the greater curvature of the stomach and proximal part of the duodenum, folding back up and attaching to the anterior surface of the transverse colon. It has a role in immunity since it contains a high number of resident inflammatory cells, such as B and T lymphocytes and it can migrate to infected viscera or to a disturbed site. The lesser omentum attaches from the lesser curvature of the stomach and the proximal part of the duodenum to the liver. It is composed by the hepatogastric ligament and the hepatoduodenal ligament [79].

The omentum has been used in surgery over the last three decades. It has been associated to bacteria inactivation and foreign materials absorption. Moreover, due to its high angiogenic capacity and neurotrophic properties, it has been related to the promotion of the recovery of a damaged tissue [79]. So, decellularized omentum from human [80], rat [80] and pigs [81–83] have been studied for different applications.

I-2.2.3. Pleura

Pleura is a transparent membrane that covers the lung and lines the walls of the thoracic cavity. The main function of pleura is to allow the optimal expansion and contraction of the lungs during breathing. This membrane is composed by two thin layers, the visceral pleura (inner layer) and the parietal pleura (outer layer). The visceral pleura wraps around the lungs and cannot be peeled off while the parietal layer lines the inside of the chest wall. Both layers are composed by a layer of mesothelial cells that secrete unsaturated and saturated phospholipids and hyaluronic acid that acts as lubricant, allowing the parietal and visceral pleura to glide over each other without friction [84,85].

Studies on pleura decellularization are scarce. Most studies focus on lung decellularization. Nevertheless, some of these works study pleura as a component of lung. So, there is some available information about decellularized pleura from mice [86], rats [87–89], humans and pigs [90].

I-2.3. Placental Membranes

The placenta is an organ that is formed during pregnancy. The human placenta is a discoid organ that has two surfaces, the chorionic plate and the basal plate. The chorionic plate faces the baby and is attached to the umbilical cord, while the basal plate abuts the maternal endometrium [91]. The placenta is the life support system for the fetus, facilitating gas and nutrient exchange between mother and fetus and also acting as barrier, protecting the fetus [92].

During pregnancy, fetus and amniotic fluid are involved by fetal membranes, the amnion and the chorion membranes. These membranes are responsible for protection but also for the exchange of nutrients and metabolic products with the maternal body [93]. The amnion or amniotic membrane is the inner membrane, being in contact with the fetus while the chorion membrane is attached to the amnion membrane in the maternal side [92]. The characteristics and composition of these two membranes will be described below.

I-2.3.1. Amniotic Membrane

The amniotic membrane is a thin translucent membrane without nerves, muscles, or lymphatic ducts. It is composed by 5 different layers, from fetus to mother: 1) Epithelium; 2) Basement membrane; 3) Compact layer; 4) Fibroblast layer; 5) Spongy layer. The epithelium is in direct contact with the amniotic fluid and consists in a single layer of flat, cuboidal and columnar epithelial cells. These epithelial cells secrete collagen type III and IV, laminins, nidogen, fibronectin and vitronectin that compose the basement membrane [92]. The compact layer together with the fibroblast layer and the spongy layer form the stroma that contributes to the tensile strength of the membrane [94].

Decellularized human amniotic membrane have been intensely studied, having three commercial products available, Biovance®, AmnioFill® and SurFactor® to be used in clinic.

I-2.3.2. Chorion Membrane

The chorion membrane is composed by three different layers: 1) Reticular layer; 2) Basement membrane; 3) Trophoblast layer. The reticular layer contacts with the spongy layer of the amniotic membrane and is composed by collagen type I, III, IV, V and VI. The basement membrane anchors the reticular and the trophoblast layers and is mainly composed by collagen type IV, fibronectin and laminin. Finally, the trophoblast layer contacts with the maternal decidua and is the most cellular layer of the chorion membrane, presenting 2-10 layers of trophoblasts [92,95].

Decellularized human chorion membrane has only been a subject of intense research in the recent years [96].

I-3. THE AVAILABILITY OF INNER BODY MEMBRANES

An important issue about decellularized inner body membranes is their availability. When a tissue engineering strategy is proposed, it is important to have access to the material that will be used. Moreover, the obtention technique should be non-invasive (or as less invasive as possible) and should not be expensive, in order to allow for the scale-up of the strategy into the clinic.

As the name implies, inner body membranes are located inside the body. This may present a problem to harvesting them. In most cases, autologous inner body membranes are used, meaning that membranes are not processed and are harvested during surgery. This leads to larger incisions and a

higher surgery time, being associated with an increased morbidity, including postoperative pain, wound complications and longer hospital stay [97]. Moreover, in some cases there is a limited amount of healthy tissue available.

To overcome these problems, cadaveric inner body membranes are used from humans or other animals (as described above), allowing an easier availability of the tissue in different sizes. A different case is the placental membranes. Since the placenta is a frequent biological waste, it is readily available after full-term pregnancies and its obtention is not associated with an invasive procedure.

When using heterogeneous membranes, an efficient decellularization is important to avoid immune-mediated implant rejection [4,15]. Moreover, ECM components are generally conserved among species, so decellularized matrix scaffolds are well tolerated even by xenogenic recipients [15,98,99]. Besides, donors have to be screened and tested for several viruses such as hepatitis and HIV, to ensure the safety of the procedure.

I-4. DECELLULARIZED INNER BODY MEMBRANES IN TISSUE ENGINEERING

Decellularized Inner body membranes have been used in tissue engineering for different applications. Depending on membrane natural function and composition they are suitable for different purposes. Some works were performed to study the potential benefit of using decellularized inner body membrane in general tissue engineering applications.

Porcine decellularized fascia was shown to maintain an intact three-dimensional architecture and strong mechanical properties. Moreover, it retained collagen, laminin, glycosaminoglycans, and vascular endothelial growth factor (VEGF), being compatible with human adipose-derived stem cell integration and proliferation. When implanted heterotopic and orthotopically in a rodent model, decellularized porcine fascia showed biocompatibility and myogenic properties. Nevertheless, when compared with decellularized porcine muscle, decellularized fascia was associated with strong mechanical properties and with less pro-angiogenic and pro-myogenic properties [27]. Porcine mesothelium was also shown to retain ECM proteins such as collagen type IV, fibronectin, and laminin. Moreover glycosaminoglycans, cytokines and growth factors (VEGF, FGF, TGF- β) were also preserved. Decellularized mesothelium stimulated human fibroblasts to produce more VEGF than fibroblast cultures on tissue culture plastic. Further works should identify the mechanism of the robust cellular growth on this material and define tissue engineering applications [100].

Composites using decellularized bovine pericardium were explored for tissue engineering applications [57,68,101]. It was shown that decellularized bovine pericardium coated with nanofibers of a blend of poly(L-lactic acid)-co-poly(ϵ -caprolactone) (PLACL) and gelatin was associated with an increase in human bone marrow mesenchymal stem cells adhesion, proliferation, and differentiation into endothelial cells, when compared with the other conditions (decellularized bovine pericardium alone and decellularized bovine pericardium coated with PLACL) [57]. It was also demonstrated that the molecular weight of urethane oligomers regulated the degree of crosslinking and its chemical composition (hexamethylene diisocyanate or isophorone diisocyanate) influenced the cytocompatibility and biodegradation of decellularized bovine pericardium, suggesting that urethane oligomers may be used as safer crosslinkers for protein-based biomaterials [101]. Furthermore, decellularized bovine pericardium crosslinked with a hyaluronic acid derivative obtained by coupling adipic dihydrazide on the -COOH of hyaluronic acid had a lower degree of calcification and lower ratios of amorphous calcium phosphates (when compared with decellularized bovine pericardium crosslinked with glutaraldehyde), indicating its ability to be used in anti-calcification bioprotheses [68]. On the other hand, human decellularized pericardium was shown to support the viability, attachment, metabolism, and proliferation of human cardiac progenitors. Moreover, it enhanced the differentiation of human cardiac progenitors into mature cardiomyocytes when compared with tissue culture polystyrene [102].

Decellularized human amniotic membrane was shown to be compatible with porcine oral epithelial cells [103], primary rabbit's smooth muscle cells [104], and to have good biocompatibility when implanted in a rabbit's uterus, demonstrating a high integration rate with rat's abdominal tissue [104]. In 2015, it was proposed a simple and cost-effective method for human amniotic membrane decellularization, using EDTA, NaOH and ammonium chloride [105]. Results shown that the decellularization did not deteriorate the mechanical properties, increased *in vitro* biodegradation rate, and did not altered membrane biocompatibility *in vitro* using rat bone marrow stem cells. Moreover, by subcutaneous implantation in a rat model, it was shown that the decellularization process slightly increased the tissue's biocompatibility *in vivo*, suggesting the absence of toxic agents in the decellularized tissue [105]. In a different work it was verified that decellularized human amniotic membrane cross-linked with genipin had better biostability and *in vitro* cytocompatibility when compared with native and decellularized human amniotic membrane. Nevertheless, genipin concentrations need to be optimized in accordance with the intended membrane application [106].

In the next sections, works using decellularized inner body membranes with specific tissue engineering applications will be described. Sections will be divided in cardiovascular applications (sub-

sectioned in myocardial reconstruction, heart valve engineering, and vascular grafts), musculoskeletal applications (sub-sectioned in bone, cartilage and tendon), skin applications, fetal membranes applications, ocular applications, *in vitro* models, and other applications that comprises clinical niches for decellularized inner body membranes application. On Table I-1 is possible to identify the main clinical tissue engineering applications of decellularized inner body membranes.

Table I-1 Summary of the clinical tissue engineering applications of the decellularized inner body membranes.

Decellularized Membrane	Application	Outcomes	References
Periosteum	Bone regeneration	Biocompatible with host cells, human umbilical cord endothelial cells and osteoblast precursor cells. It is associated with and efficient osteogenic activity, angiogenesis and mineralization.	[31,33,35,107]
	Cartilage regeneration	Seeded with autologous chondrocytes has shown to be capable of cartilage regeneration.	[108,109]
Synovial Membrane	Cartilage regeneration	Able to deliver anabolic genes locally.	[37]
Pericardium	Myocardial infarction	Enhances the proliferation, viability and migration of cardiac progenitor cells and adipose mesenchymal stem cells. Neoformation of vessels and nerves.	[110–114]
	Heart valve engineering	Avoids calcification <i>in vivo</i> and supports host cell infiltration, matrix remodeling, and endothelialization. It has long-term structural stability.	[49,60,115,116]
	Vascular grafts	Well preserved <i>in vivo</i> with a monolayer of endothelial cells in the luminal side and a well-developed neo-vascularization in the outer side. No evidence of calcification.	[51]
	Bone regeneration	Enhances the deposition of mineralized tissue.	[52]
	Tendon regeneration	Biocompatible with human sheath synoviocytes and human adipose derived stem cells. Promotes human adipose derived stem cells differentiation and hyaluronic acid production by both cell types.	[46]
Peritoneum	Skin regeneration	Associated with a thicker epidermis and dermis.	[72–74]
Mesentery	Vascular grafts	Reconstruction of a rich vascular network with several segmentally radiating branches.	[77]

Omentum	Myocardial infarction	Biocompatible with cardiac cells and associated with a quick vascularization <i>in vitro</i> .	[81]
	Myocardial infarction	Prevents pathologic postinfarct remodeling processes, improving systolic and diastolic function.	[117]
	Vascular graft	Promotes enhanced and early cell remodeling and improves blood perfusion.	[118–120]
	Bone regeneration	Acts as a shield to avoid fibrous tissues invasion and stabilizes bone grafts.	[121]
	Cartilage regeneration	Promotes hyaline cartilage repair.	[122]
Amniotic Membrane	Tendon regeneration	Enhances the mature organization of fibroblasts and collagen fibers and modulates the inflammatory response in tendon wound healing.	[123,124]
	Skin regeneration	Presents biocompatibility, good mechanical properties, low immunogenicity and some antibacterial properties.	[125–133]
	Fetal membranes	Promotes abundant re-epithelization, minimal local inflammation and consequently, fetus survival.	[134–136]
	Ocular regeneration	Supports the growth of human limbal epithelial cells, immortalized corneal epithelial cells, and induced pluripotent stem cells.	[137–141]

I-4.1. Cardiovascular applications

Cardiovascular diseases are the leading cause of death in industrialized countries, and it is rising in developing countries [117,142]. Cardiovascular diseases involve the heart or blood vessels. Symptoms may be alleviated by medication. Nevertheless, this is associated to several side effects. Taking into consideration that is more beneficial to treat the root of the problem, several studies have been focused on the manufacture of new patch materials with potential advantages for vascular and heart repairs [47]. In this section, we will describe the use of decellularized inner body membranes for cardiovascular applications.

Decellularized inner body membranes such as pericardium, omentum or amniotic membrane have been described for general cardiovascular applications. Decellularized porcine pericardium was demonstrated to be non-cytotoxic by extract analysis (ISO 10993/5) and mouse subcutaneous assay and to have a good compatibility with human endothelial progenitor cells [47]. Bovine decellularized pericardium was also demonstrated to be biocompatible with bovine aortic endothelial cells and to maintain the biochemical and biomechanical properties of the native tissue [58]. These results were corroborated in a study comparing decellularized human fascia lata, human pericardium, porcine pericardium and porcine peritoneum for cardiovascular applications [23]. On the other hand, five different decellularization protocols of porcine omentum were studied regarding the elimination of cellular components, the maintenance of the native ECM and the biocompatibility with mouse fibroblast and rat cardiomyocytes [81]. Decellularized amniotic membrane was coated with ECM derived from human myocardium, showing *in vitro* compatibility with murine cardiomyocytes, human cardiac fibroblasts, immune cells and epicardial derived-cells [142]. The decellularized amniotic membrane was also used to mimic the pericardium in rats. The results of the study demonstrated that decellularized amniotic membrane was associated with a low immunogenicity and to a lower occurrence of adhesions and scarring and also to a thicker pericardium [143].

In the next section, the application of decellularized inner body membranes in specific cardiovascular applications, such as myocardial reconstruction, heart valves engineering and vascular grafts, will be described.

I-4.1.1. Myocardial reconstruction

Myocardial infarction is associated with a high mortality, morbidity and economic burden [144]. Fibrotic remodeling, scar formation, and collagen disposition play a key role in infarcted myocardium repair [145]. Moreover, the pool of cardiac progenitor cells is limited and adult cardiomyocytes are unable to proliferate [146]. So, myocardial infarction is associated with myocyte loss and scar formation.

Myocardial infarction management has considerably evolved in the past 50 years, with the incorporation of aspirin, β -blockers, angiotensin-converting enzyme 1, and the use of coronary stents. As innovations emerged, the residual scar has become smaller. However, there is still a portion of the heart that is not fully recovered [110]. Moreover, there is also a shortage of cardiac donors [81]. This scenario motivated the exploration of new technologies for heart regeneration, using tissue engineering and progenitor cells as complementary or alternative to conventional therapies [81,110].

The use of decellularized tissues is one of the strategies to treat damaged myocardium, through the fabrication of myocardial patches [113]. In general, myocardial patches or scaffolds, should be able to mimic the structural architecture of the cardiac muscle, to be deformable to support cardiac contraction, to promote cell migration, and to promote the vascularization of the newly formed tissue [110]. Regarding inner body membranes, due to its location and function, pericardium is the most adequate membrane to be used in myocardial regeneration applications [147].

Along the years, it was developed the concept that three-dimensional (3D) ECM-like scaffolds were crucial for cardiac tissue engineering [81]. Following this tendency, it was developed a scaffold using human decellularized pericardium filled with a mixture of RAD16-I hydrogel and porcine mediastinal adipose tissue-derived progenitor cells, together with an implantable EIS measurement system for *in vivo* online monitoring of scar formation in a swine model of myocardial infarction [110]. This work, together with another study of the same group [111], allowed the creation of a new myocardial bio prosthesis with online monitoring of cardiac repair. This system allows a noninvasive tracking of myocardial scar healing [110,112]. Moreover, it was demonstrated that the same pericardium-based scaffold was associated with the neoformation of vessels and nerves when implanted in the same swine model [111]. Functional vasculature and innervation are important to produce viable and stable contractile function [111]. Rajabi-Zeleti, *et al*/ also developed a 3D cardiac scaffold based on human decellularized pericardium. They demonstrated that their 3D macroporous scaffolds enhanced the proliferation, viability, migration and proliferation of human cardiac progenitor cells *in vitro* and angiogenesis and cell differentiation *in vivo*, when compared to decellularized pericardium and collagen 3D scaffolds. Moreover, no xenogeneic

immune reaction was observed one month after subcutaneous implantation in rats [113]. Another strategy proposed to repair the myocardium was the use of an injectable pericardium scaffold. In this work, two injectable pericardium scaffolds (porcine and human) were studied for its biochemical composition, *in vitro* chemoattractant potential and *in situ* cellular infiltration, proving the concept of using decellularized pericardium matrix gels as injectable scaffolds for myocardial treatment [148].

Recently, decellularized rabbit pericardium was used in a rabbit myocardial infarction model. In this study, it was hypothesized that the decellularized pericardium alone or seeded with autologous adipose derived mesenchymal stem cells, could be safely used. In fact, it was demonstrated that when compared with the control condition (no intervention), both pericardium alone or seeded, improved left ventricular contractile function and myocardial pathological changes. Nevertheless, pericardium seeded with adipose derived mesenchymal stem cells showed to be better in scar repairing two months after surgery [114]. This was already expected since it was demonstrated that human mesenchymal stem cells and cKit⁺ cardiac stem cells could reduce scar size and improve the cardiac function after myocardial infarction [149].

Other decellularized inner body membranes have been studied for myocardial repair, such as omentum and amniotic membrane [81,117]. Porcine omentum was shown to support the cultivated rat cardiac cells *in vitro*. Moreover, due to the dense vascular network that characterized the omentum, a quick vascularization of the patch was achieved *in vitro* using human umbilical vein endothelial cells [81]. Decellularized human amniotic membrane was used in a mice model of myocardial infarction. The effects of decellularized human amniotic membrane and cellular amniotic membrane with or without epithelial to mesenchymal transition (EMT) induction on postischemic left ventricle were studied. It was found that although the gross infarct size remained unchanged, the decellularized amniotic membrane attached to the surface of the acutely infarcted left ventricle and helped preventing pathologic postinfarct remodeling processes, improving systolic and diastolic function. Moreover, cellular amniotic membrane (with or without EMT) elicited a local immune response [117].

Diverse strategies using different decellularized inner body membranes have been proposed for myocardial repair. Nevertheless, studies comparing different decellularized inner body membranes for myocardial repair would be important to fully understand the advantages and disadvantages of some membranes over others.

I-4.1.2. Heart valves engineering

An ideal tissue-engineered heart valve should accomplish some basic requirements. It should allow for cell migration and tissue development *in vivo*. Moreover, cells seeded in the prosthetic valve before implantation (preferably autologous), should lead to further growing of a supporting matrix [60]. Decellularized pericardium fulfills many properties required for its use in valvular tissue engineering, including adequate mechanical properties, minimal cytotoxicity, excellent repopulation potential and propensity for matrix remodeling [115]. Moreover, the pericardial tissue is easy to manipulate and shows durability *in vivo* [49].

Decellularized porcine pericardium is widely used in valve engineering. Collagen-based scaffolds from decellularized porcine pericardium were created. Collagen scaffolds are superior to polymer scaffolds in terms of mechanical and biological properties but also maintain a natural tendency to degenerate rapidly *in vivo* if they are not stabilized [115]. Different cross-linking protocols to stabilize the collagen were tested. Collagen-based scaffolds derived from decellularized porcine pericardium, were treated with penta-galloyl glucose (PGG) and glutaraldehyde. While, glutaraldehyde cross-linking fully stabilized collagen, it did not allow tissue remodeling and induced calcification when implanted subdermally in rats. On the other hand, PGG cross-linking, was able to stabilize collagen, avoiding calcification *in vivo* and supporting host cell infiltration and matrix remodeling [115].

Pericardium treated with glutaraldehyde was the material of choice employed in the manufacture of commercial bioprosthetic valves. However, this was associated with severe side effects resulting from the lack of biological compatibility. So, protocols using decellularized porcine pericardium without the use of fixatives appeared, showing compatibility with both porcine and human aortic valve interstitial cells [150]. Following this rationale, other studies exploring methods to seed cells in bioprosthetic valves were developed [48,151].

The impact of porcine pericardium processing, especially sterilization (using supercritical CO₂) on the material's mechanical properties, was reported. Decellularized pericardium, fixed pericardium, and decellularized-sterilized pericardium were compared with native valvular and pericardial tissue. The decellularized tissue showed the lower mechanical properties. However, the decellularized-sterilized condition demonstrated elastic properties similar to the average porcine aortic cusp. Moreover, decellularized-sterilized tissue was able to accept host-derived cells, allowing surface endothelization and long-term structural stability [49]. Religious and cultural practices may limit the use of explanted tissues. For example, one patient may not accept the use of a porcine bioprosthetic. Therefore, there is a need to

characterize tissues from different sources in order to fit the needs of the patients [152]. So, the behavior of human vascular cells from saphenous vein seeded on bovine decellularized pericardium, both *in vitro* and *in vivo*, were studied. *In vitro*, the seeded cells showed increase of endothelial activity from day 1 to day 7 and remained stable until day 21. For the *in vivo* assay, the decellularized bovine pericardium seeded with human cells was implanted subcutaneously in nude mice. It was demonstrated that after 30 days, the cells could differentiate and showed all the identities of human endothelial cells, smooth muscle cells, and fibroblasts. These results suggest that human vascular cells from the saphenous vein are an optimal cell source for seeding on decellularized bovine pericardium scaffold for construction of tissue-engineered heart valves [60]. Moreover, others reported a case of replacing the mitral valve of a neonate using a cylindrical prosthesis fashioned from decellularized equine pericardium. A good performance of the mitral valve prostheses at four months of follow up was observed. However, since there is no evidence of the growth potential of cylindrical prosthesis using natural materials, a subsequent valve replacement will be necessary in growing children [116].

I-4.1.3. Vascular grafts

Vascular deformities are usually caused by vascular diseases, congenital defects, trauma or tumors [153]. The use of autologous blood vessels is limited not only by the availability of the donor but also by the lack of healthy vessels. So, tissue engineered substitutes for blood vessels have been widely studies.

Xenograft substitutes have attracted considerable attention. A study used decellularized equine pericardium, implanted it in the descending aorta of juvenile sheep [51]. After 4 months, the grafts were well preserved with a monolayer of endothelial cells covering its luminal side. The outer side of the patch was characterized by a well-developed neo-vascularization. Host fibroblasts were present in all layers of the scaffold. Moreover, there was no evidence of structural deterioration or calcification of the scaffolds [51]. Nevertheless, after vascular graft implantation, it is important to ensure blood supply. A major barrier to a successful vessel graft substitute is the need of a functional vascular network within the engineered tissue. To solve this problem, a novel vascular scaffold was established from decellularized porcine aorta and intact mesentery containing a radiating arterial and venous network [77]. Since the mesentery is rich in small arteries and veins that spread for the outside, it can directly provide blood to an area of ischemic tissue. So, this scaffold provided vascular channels for blood supply. When transplanted into dogs, the aorta of the scaffold was linked to the femoral artery of the dog by arterial anastomoses, creating a rich

vascular network with several branches. Consequently, this approach was presented as a potential alternative for the xenogeneic transplantation of vessels, compensating the lack of allogenic tissues available for transplantation [77].

Allogenic grafts for vessel replacement are based in the use of human amniotic membrane. This membrane has been used as a single layer with omental microvascular endothelial cells. The use of sphingosine 1-phosphate (S1P) treatment allowed omental microvascular endothelial cells to form a capillary structure on the decellularized amniotic membrane. The scaffold was transplanted into ischemic hindlimbs of mice and the capillary structure significantly improved the blood perfusion 5 days after implantation [118]. Other studies using the human amniotic membrane involve rolling it in a tubular construct. These studies demonstrated that the rolled scaffold provided a 3D microenvironment that resembled natural arteries, promoting an enhanced early cell remodeling [119,120].

The advantage of using human amniotic membrane for the construct of vascular grafts is associated with its greater availability when compared with equine pericardium or porcine mesentery.

I-4.2. Musculoskeletal applications

The human musculoskeletal system is an organ system that provides shape, support, stability, and articulation of movements to the body. It is composed of bones, muscle, cartilage, tendons, ligaments, and joints. In this section the use of decellularized inner body membranes for musculoskeletal tissue engineering applications will be explored, mainly for bone, cartilage, and tendon.

I-4.2.1. Bone

Bone defects may be caused by trauma, tumor resection, pathological degeneration, or congenital deformations. An insufficiently treated bone defect can compromise the future mobility of the patient, especially in load-bearing areas. The healing capacity of bone is limited, so critical-sized bone defects may not heal by themselves, resulting in a nonunion [31]. In these cases, bone grafting or bone substitutes are typically used [33]. Autografts are the gold standard for the repair of bone defects. However, its clinical application is limited due to the donor site morbidity and the amount of tissue that can be obtained [31,33].

Periosteum is important for the initiation of bone graft healing and remodeling due to its regenerative capacities [33]. So, various studies explored the use of periosteum for bone regeneration.

Rabbit decellularized periosteum was shown to allow rabbit periosteum cells to adhere, proliferate and infiltrate *in vitro*. Moreover, its biocompatibility was demonstrated in an *in vivo* subcutaneous assay in rabbits [33]. Another study demonstrated the *in vitro* mineralization using pig decellularized periosteum and that the same scaffold could effectively promote orthotopic regeneration in a critical size bone defect rabbit model [107]. Decellularized rabbit tibial periosteum was compared with decellularized rabbit calvarial periosteum regarding their biological, structural and biomechanical properties, its cytotoxicity, and angiogenic activity (using chicken egg chorioallantoic membrane assay). Osteogenic activity was also studied by the subcutaneous implantation of the decellularized periosteum seeded with mesenchymal stem cells in nude mice. Moreover, angiogenesis and osteogenesis mechanisms were analyzed by seeding human umbilical vein endothelial cells and mouse osteoblast precursor cells, respectively, onto the decellularized scaffolds [35]. In summary, rabbit decellularized calvarial periosteum was associated with a stronger angiogenic activity while rabbit decellularized tibial pericardium was associated with a more marked osteogenic activity [35]. Thus, it was proposed the use of decellularized calvarial periosteum in children with incomplete ossification and the use of decellularized tibial periosteum to promote the repair of bone defects or injuries in adults [35]. In a different approach, a hybrid material combining decellularized rabbit periosteum with chitosan globules was studied [31]. It was expected a synergistic effect between the two materials, maintaining the biocompatibility and biodegradability while boosting the overall mechanical stability. The efficacy of the new scaffold was assessed using a rabbit femoral bone defect model. Chitosan-decellularized rabbit periosteum shown to have a more effective surface for cell adhesion and a superior performance for osteogenesis in the defect area when compared with chitosan globules alone or with the empty defect [31].

Guided bone regeneration was developed to enhance the efficacy of bone repair [52] by the employment of a barrier membrane to protect the space necessary for osteogenesis and to prevent the invasion of fibroblasts. It has been widely used in the dental peri-bone implantation and other bone fractures with large bone loss or poor healing potential. The main goal of applying a barrier membrane is to prevent the invasion of fibroblasts, provide stability for bone grafts and blood clots, and ensure a protected space for osteogenesis to occur [121]. Expanded polytetrafluoroethylene has been extensively studied as a barrier membrane. However, this material is not absorbable, requiring a second surgical procedure for removal, which is time-consuming, expensive and increases patient's discomfort and risk of secondary infection [154,155]. As an alternative, collagen membranes were proposed as resorbable membranes. Nevertheless, due to *in vivo* degradation, collagen is structurally unstable [52]. Decellularized inner body membranes were also investigated for this application. Decellularized bovine

pericardium has shown *in vivo* efficacy in a rabbit mandibular model. After 16 weeks of *in vivo* implantation, this membrane was associated with an enhanced deposition of mineralized tissue when compared with defects left empty for spontaneous heal [52]. Moreover, a study using a lyophilized multilayered decellularized human amniotic membrane showed its functionality as a membrane barrier for guided bone regeneration in a rat tibia defect model. After 6 weeks of surgery, the membrane acted as a shield to avoid the invasion of the fibrous tissues, to stabilize bone grafts and to induce massive bone growth (when compared with collagen membranes) [121].

In summary, decellularized periosteum from rabbits and pigs were shown to promote bone regeneration. However, periosteum harvesting site influences its angiogenic and osteogenic properties. For guided-bone regeneration, when compared with decellularized bovine pericardium, decellularized human amniotic membrane presents advantages such as its availability and allogenic source (when for human use).

I-4.2.2. Cartilage

Cartilage is a connective tissue that covers and protects the ends of long bones at the joints and nerves and is present in the ends of the ribs, separating each vertebra in the spine, ears, nose and bronchial tubes. Cartilage degeneration or injury is mainly caused by osteoarthritis and traumatic injuries and may lead to progressive deterioration and eventual osteoarthritic degeneration. However, cartilage repair is very limited due to its low regenerative capacity [122].

Autologous chondrocyte implantation was previously explored as treatment option for cartilage lesions [108,109]. The use of decellularized periosteum seeded with autologous chondrocytes has shown to be capable of regenerating the articular cartilage of the rabbit patella [108]. Moreover, the use of human chondrocytes with decellularized periosteum demonstrated 73% hyaline-like cartilage regeneration in femoral condylar injury in a cohort of 23 human patients [109]. However, the implantation of autologous chondrocytes with periosteum has been associated with various complications, such as large surgical incisions, peripheral graft hypertrophy, graft delamination, possible calcification and the ability of periosteum to form ectopic bone [156]. These complications stimulated the development of alternative scaffolds for autologous chondrocyte implantation, including membranes of purified porcine collagen type I and III [156–158]. Autologous chondrocyte implantation produces good short-term results, but there are limitations regarding the cost, the need for a two-step procedure (cell collection and re-implantation after cells expansion) and the difficulty of using this technique in larger defects [122]. So, acellular

scaffolds were studied for cartilage repair. Since cartilage repair mediated by cell-free scaffolds depends on the *in situ* recruitment of endogenous cells, the acellular scaffold needs to allow host cell recruitment, attachment, growth and differentiation [122]. ECM from mesenchymal stem cells derived from the human amniotic membrane was combined with polylactic-co-glycolic acid (PLGA) to create a scaffold for cartilage repair. This acellular scaffold was implanted into osteochondral defects in the trochlear groove of rat knees and promoted the in-growth of endogenous cells that induced gradual tissue regeneration, resulting in hyaline cartilage repair that was not observed in the empty control group [122]. Another therapeutic strategy for cartilage repair using decellularized inner body membranes is the delivery of anabolic genes locally. To study this hypothesis, decellularized synovial membrane from the femoropatellar and medial femorotibial joints of equine cadavers was seeded with synovial-derived mesenchymal stem cells (from the same sites) [37]. In this work, seeded synovial-derived mesenchymal stem cells were not treated or were cotransduced to enhance the expression of green fluorescent protein (GFP) and human bone morphogenetic protein (BMP)-2. Results demonstrated that equine decellularized synovial membrane seeded with untreated or transduced synovial-derived mesenchymal stem cells resulted in a bioactive scaffold, leading to cell viability, incorporation into the scaffold, and differentiation. Moreover, transduced cells produced greater BMP-2, hyaluronic acid and proteoglycan concentrations, indicating gene expression and enhanced synovial function, promoting cartilage repair [37].

Decellularized inner body membranes such as periosteum or synovial membrane have been used in cartilage repair to support the delivery of cells and/or genes to the injury site. However, further studies are needed to better understand the therapeutic potential of ECM based acellular scaffolds in cartilage regeneration.

I-4.2.3. Tendon

Tendons are tough fibers that connect muscles to bone. Most tendon injuries occur near the joints at the interface with bone. It may occur suddenly, but it is usually associated with gradual wear and tear to the tendon from overuse or aging.

ECM scaffolds and tendon grafts were studied for the repair of tendon ruptures, including Achilles tendon, rotator cuff, and flexor tendons [159,160]. Materials derived from biological sources seem to be more promising for tendon repair since they are associated with a faster and more natural healing of tendon defects using host cells, and with an adequate resorption rate [160]. Decellularized bovine amniotic membrane was used on a rupture Achilles tendon model in rabbit [124]. Biomechanical and

histological analyses were performed. It was observed that the amniotic membrane enhanced the development of a mature organization of fibroblasts and collagen fibers and improved the properties of the lacerated tendon at the early healing period, when compared with the control group (no treatment). In this study, the long-term effects of the decellularized bovine amniotic membrane on tendon healing were not investigated [124]. However, tendon healing following injury, even when surgically repaired, results in a fibrocartilaginous scar that leads to a decrease in tendon strength and disorganized collagen fibers [123]. In the uninjured tendon, the tendon sheath is a thin synovial membrane that separates the tendon from its surrounding tissue and is composed by synoviocytes and a sheath of fibroblasts. The main purpose of these cells is to produce synovial fluid (mainly hyaluronic acid) that provide nutrition to the tendons and ensure a smooth gliding surface [161]. If the tendon and its sheath are injured, this natural barrier is lost and may lead to postoperative adhesions and scarring. Nevertheless, the human tendon sheath is not possible to be harvested from cadavers due to its brittle consistency and firm attachment to the surrounding bone. Thus, decellularized inner body membranes have been studied for this purpose. The decellularized porcine pericardium was shown to be compatible with human sheath synoviocytes and human adipose derived stem cells. After seeding, adipose-derived stem cells demonstrated a shift in gene expression by down-regulating *collagen type I* and *III* and up-regulating the production of *hyaluronan synthase 2*. After 8 weeks, a continuous production of hyaluronic acid by both cells was observed [46]. The capacity of the decellularized human amniotic membrane to modulate the inflammatory response in tendon wound healing was also studied. This work combined collagen-glycosaminoglycan scaffolds with hyaluronic acid or decellularized amniotic membrane. The combined scaffolds normalized the expression of genes associated with inflammatory response in normal tendon healing (*TNF- α* , *COL I*, *MMP-3*). Moreover, the use of decellularized amniotic membrane was associated with increased mechanical properties and increased metabolic activity even when media was supplemented with the pro-inflammatory cytokine interleukin-1 β [123].

Late tendon healing phases are of main importance since they determine the presence of fibrocartilaginous scars associated with a decrease in tendon strength and functionality. Taking into account the anti-inflammatory properties of the decellularized amniotic membrane and its ability to promote tendon regeneration in early healing stages, this may be a good substrate for the development of further tendon healing therapies.

I-4.3. Skin applications

Skin is the largest protective organ of the human body and plays an important role in defending the organism against harm from the external environment [162]. So, any skin wounds such as burns, chemical damage and tearing apart are associated with barrier function loss and may lead to body infections, ulcers and ultimately scars after wound healing [125,131]. The progress of wound healing repair is complex, continuous and dynamic. It comprises three phases: inflammation, proliferation, and remodeling. All these phases involve several cells, cytokines and growth factors which interact with each other to regenerate the skin layers [131].

Traditional methods for wound healing include autologous skin grafting, skin flap transplantation, and allogeneous or xenogeneic skin transplantation. The application of these strategies is limited due to donor availability, immunological rejection and the possibility of disease transmission [163]. So, there is a need for skin substitutes particularly in large and life-threatening wounds, such as skin burns [125].

The human amniotic membrane was considered for wound healing applications due to its availability, structure, biological and mechanical properties and low immunogenicity [164]. Several studies demonstrated the value of decellularized human amniotic membrane in wound healing, using different strategies [125,126,128–133,165]. A 3D micronized decellularized human amniotic membrane scaffold was developed to act as a microcarrier able to prevent human epidermal stem cells differentiation. This study demonstrated that the scaffold could effectively retain the stem cells' characteristics. Moreover, when implanted in a full thickness skin defect model using nude mice, human epidermal stem cells survived well and formed a new epidermis, papilla-like structures were detected, and regularly arranged collagen fibers were observed in the newly formed dermal layer [165]. Another work crosslinked the decellularized human amniotic membrane with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and seeded it with human epidermal keratinocytes. This scaffold was grafted onto a full-thickness skin defect model in nude mice, demonstrating an epidermis formation similar to normal skin, reconstruction of the dermal structure with an intact basement membrane, and low wound contraction rate [132]. A different approach followed was to develop a decellularized human amniotic membrane scaffold activated with platelet rich plasma (PRP) and calcium chloride for burn wound dressing in a mice model. After seven days, results demonstrated that this new scaffold outperformed the other test groups (silver nitrate gel dressing and natural healing), showing an almost closed wound without scarring, with a well differentiated epidermis, keratinocytes proliferation, hair follicles, and basement membrane [128]. Nevertheless, there are several works using the decellularized human

amniotic membrane without any treatments. In 2014, the human decellularized amniotic membrane was combined with human Wharton's jelly mesenchymal stem cells, showing its effectiveness in the treatment of a skin injury of severe combined immunodeficient (SCID) mice [133]. Moreover, the decellularized human amniotic membrane was also seeded with human adipose-derived mesenchymal stem cells, showing a facilitated healing of nude mice full-thickness skin wound when compared with cellular human amniotic membrane and decellularized human amniotic membrane alone. Additionally, it was also associated with a phenomenon of hair follicle development during the process of skin repair [125]. In 2017, it was explored the mechanism by which the decellularized human amniotic membrane accelerated and strengthened the process of wound healing in a rat full thickness wound model. It was demonstrated that the decellularized human amniotic membrane increased the expression level of VEGF (important in the proliferation phase of wound healing) and alpha-smooth muscle actin secretion (important in the remodeling phase of wound healing). Moreover, it alleviated inflammation and relieved scar formation by decreasing excessive TGF- β 1 levels (inflammation phase) [131].

The prevalence of antibiotic-resistant bacteria is increasing, especially in developing countries, due to the overuse of antibiotics. So, the development of multidrug-resistance bacteria strains is a major concern in the health care of burned patients [166]. The antibacterial activity of both cellular and decellularized human amniotic membrane was also investigated. For that, resistant bacteria were isolated from burns, identified and tested for their susceptibility to antibiotics. Moreover, the antibacterial activity of human amniotic membrane was also tested using commercial bacterial strains. Results showed that the human amniotic membrane only had antibacterial activity against three commercial bacterial strains (*Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*). This data suggested that a complementary strategy is needed to the use of human amniotic membrane in wound grafting in order to fully protect burns from nosocomial infections [126]. Radiation wounds are associated with radiotherapy and occur in 60% of surgical patients [167]. So, lyophilized decellularized human amniotic membrane seeded with rat bone marrow mesenchymal stem cells was tested in a rat radiation wound model. No differences were observed between using decellularized human amniotic membrane with rat bone marrow mesenchymal stem cells lyophilized or not, suggesting that the administration of freeze-dried bone marrow mesenchymal stem cells paracrine factors together with decellularized amniotic membrane is efficient in radiation wound treatment. Moreover, lyophilization increases the shelf-life of the scaffolds, facilitating its clinical use [130].

Decellularized porcine peritoneum has also been studied for skin wound healing. In these studies, the decellularized porcine peritoneum was loaded with hyaluronic acid (HA) and epidermal growth factor

(EGF) or basic fibroblast growth factor (bFGF). It was demonstrated that the presence of HA increased the adsorption of EGF or bFGF into decellularized peritoneum and sustained its release from the scaffolds. Moreover, the use of this combined scaffolds (decellularized porcine peritoneum with HA and EGF or bFGF) was associated with a thicker epidermis and dermis when compared with the other test groups (vaseline oil gauze, decellularized porcine peritoneum alone, decellularized porcine peritoneum with EGF or bFGF) [72–74].

Further studies, comparing the different approaches using decellularized human amniotic membrane for wound healing would be of extreme importance, in order to avoid studies' bias and allow the identification of the best wound healing strategy.

I-4.4. Fetal membranes applications

Minimally invasive fetoscopic surgery was developed as an approach to treat congenital fetal malformations [168]. However, the fetoscopic procedure is associated with mechanical rupture of fetal membranes, leading to serious complications when not controlled [134]. Iatrogenic preterm premature rupture of membranes (IPPROM) occurs in approximately 1 – 2% of patients after amniocentesis, 3 – 5% after diagnostic fetoscopy, and 5 – 8% after operative fetoscopy [135], leading to an increased risk of chorioamnionitis, preterm birth, perinatal mortality, and survivor's morbidity. Moreover, spontaneous preterm rupture of membranes can also occur [169]. Although membranes may seal spontaneously, patients may continue to leak fluid and are at risk of pregnancy loss [134].

An appealing strategy for closure of iatrogenic puncture wounds in fetal membranes is to deploy biopolymeric plugs that immediately seal the membrane [135]. It was demonstrated that the decellularized amniotic membrane (from rabbit and human origin) was able to function as a plug to seal iatrogenic fetal membrane defects. The use of the decellularized amniotic membranes was associated with an abundant re-epithelization and minimal local inflammation, restoring the fetal membrane integrity in an IPPROM rabbit model [135,136]. However, studies on rabbits should be carefully interpreted since they cannot provide a full understanding of wound healing of fetal membranes due to rabbit's short gestation period (around 30 days) [135]. A study performed in an IPPROM porcine model, showed that a new 3D-printed device composed of decellularized human amniotic membrane-derived hydrogel with polycaprolactone outperformed the other test groups regarding surgery time, membrane regeneration at the defect site, and fetal survival rate [134]. However, there is still no proven method to reduce the incidence of IPPROM in humans [134].

I-4.5. Ocular applications

The ocular surface is composed by the cornea, conjunctiva, eyelids and lacrimal glands. Any disorder in these structures is classified as an ocular disorder [170]. Decellularized human amniotic membrane was widely studied to be applied in ocular disorders. When compared with cryopreserved human amniotic membrane, decellularized human amniotic membrane is associated with a loss of collagen type IV and VII, fibronectin, and with lower levels of several growth factors. However, its clinical transplantation in a patient with chronic bullous keratopathy and ocular surface instability was successful with rapid re-epithelialization [171].

Corneal blindness is the second most frequent debilitating ocular disorder, affecting 6-8 million people worldwide. Although some corneal diseases are treatable with drugs or surgery, others are difficult to treat even with corneal transplantation due to limbal epithelial stem cells (LESC) deficiency (LSCD) [172]. In the human cornea, LESC reside at its periphery and continuously renew the corneal epithelium [173]. LESC loss can result from congenital aniridia, Stevens-Johnson syndrome, chemical and thermal burns, ocular cicatricial pemphigoid, chronic inflammation, microbial infections, and extended contact lens wear [137,140]. LSCD is associated with corneal erosions and vascularization, conjunctival ingrowth, and scarring which lead to loss of corneal transparency and consequently, vision loss [174]. Transplantation of LESC culture on decellularized human amniotic membrane has emerged as a promising approach to manage LSCD. It was proposed a standardized method to decellularized the human amniotic membrane for its use in LSCD. It was shown that the suggested method, using NaOH treatment also worked on glycerol-cryopreserved human amniotic membrane, maintaining it compatible with the growth of human limbal epithelial cells, immortalized corneal epithelial cells, and induced pluripotent stem cells. Altogether, the results supported the use of this method to standardize decellularized human amniotic membrane preparations for expansion of limbal stem cells *in vitro* before transplantation to patients [137]. Human decellularized amniotic membrane cross-linked with UV radiation showed to support the proliferation of human corneal limbal cells [138]. A new composite membrane consisting of a bioabsorbable polymer electrospun fiber meshes bonded to a decellularized human amniotic membrane through interfacial conjugation was also developed for LSCD treatment. Three different polymers were used, PCL, poly(lactic acid) (PLA), and polylactic-co-glycolic acid (PLGA). This approach increased the mechanical properties of decellularized human amniotic membrane while maintaining its bioactivity in supporting rabbit LESC attachment, growth and maintenance, and significant anti-inflammatory capacity [141].

LESCs can only be expanded for several passages *in vitro* and a potential contamination from mouse 3T3 feeder cells used for LESCs culture is a major concern. Moreover, in bilateral LSCD patients do not have autologous LESCs and can only receive transplanted allogenic LESCs [175]. Thus, renewable and standardized LESCs sources are needed. One approach to solve this problem is the use of human induced pluripotent stem cells (iPSCs) that can self-renew for several passages due to the high levels of telomerase expression, allowing continual propagation, banking, and easy cryopreservation [175,176]. Decellularized human amniotic membrane and human de-epithelialized corneas, were used to direct iPSCs, derived from LESC cultures, to limbal differentiation. It was demonstrated that limbal epithelium-derived iPSCs differentiated into limbal-like cells more readily than fibroblast-derived iPSCs when culture in both membranes. Thus, such iPSCs could become a new expandable and bankable LESC source for transplantation to patients with LSCD [140].

Although decellularized amniotic membranes are widely used as corneal epithelial tissue carrier in reconstruction surgery, they are associated with a low transparency due to the translucent thick underlying amniotic membrane stroma. So, the construction of an ultra-thin decellularized human amniotic membrane using collagenase IV to thin the stroma to about 30 μm was proposed. It was demonstrated that this ultra-thin decellularized human amniotic membrane was associated with a more transparent and thicker sheet of rabbit corneal epithelial cells (generated by the expansion of limbal epithelial cells) when compared with decellularized human amniotic membrane. Moreover, in a rabbit total LSCD model, the cornea grafted with ultra-thin decellularized human amniotic membrane showed higher transparency and more stratified epithelium than the cornea grafted with decellularized human amniotic membrane-based cells sheets [139].

I-4.6. *In vitro* models

In vitro models are very important in the fields of medicine and biology, since they provide insights of cells and systems behavior. However, these cells and microorganisms are isolated from their natural environment, so, these models may not completely or precisely predict the effects on the entire organism. Nevertheless, *in vitro* models are the starting point in biological and medical research and have an important role in drug screening, providing cost-effective and satisfactory results [177].

Decellularized inner body membranes were used for the creation of *in vitro* models. The decellularized human amniotic membrane was used as scaffold to create an *in vitro* model of skin. Basically, human keratinocytes were seeded on the epithelial side of the decellularized human amniotic

membrane and human fibroblasts were seeded on the chorionic side. Results shown that the human decellularized amniotic membrane supported cells' adherence, allowing the achievement of an integral and intact skin *in vitro* model, supporting the potential use of patients own cells to develop dermis and epidermis [178]. Moreover, mice decellularized mesentery basement membrane was used in a cancer invasion *in vitro* model with a co-culture with a human colon cancer cell line and cancer-associated fibroblasts. By using this model, it was possible to understand the role of cancer-associated fibroblasts in cancer cell invasion through basement membrane rupture. It was demonstrated that cancer-associated fibroblasts facilitated basement membrane breaching in a matrix metalloproteinase-independent way, by exerting contractile forces that alter the organization and the physical properties of the basement membrane making it permissive for cancer cell invasion [76].

In summary, there is a great potential in using decellularized inner body membranes for the creation of *in vitro* models, mainly because they can mimic the *in vivo* ECM architecture, composition, and permeability.

I-4.7. Other applications

Decellularized inner body membranes have been used in other tissue engineering applications.

The decellularized human amniotic membrane was used in periodontal regeneration. For that, decellularized human amniotic membrane was seeded with human periodontal ligament stem cells. It was demonstrated that the single layer of human periodontal ligament stem cells was stable into the decellularized human amniotic membrane even with the movement and deformation caused by surgical instruments. Moreover, after implantation of the seeded scaffold into a surgically created periodontal defect in nude rat's maxillary molars, regeneration of the periodontal tissue was enhanced [179]. Moreover, decellularized lyophilized human amnion/chorion membrane was used for the treatment of pharyngocutaneous fistulas. Pharyngocutaneous fistulas is a common complication of laryngectomy. It was shown, in a cohort of 67 human patients, that the average time for the full healing of the wound using decellularized lyophilized human amnion/chorion membrane was 18 days. Comparing with other pharyngocutaneous fistula treatment, this approach avoids invasive procedures, preventing donor morbidity, and allows the management of the wound without using classical wound gauze [180].

Furthermore, commercial decellularized human fascia lata (Tutoplast®) was compared with autologous fascia lata in the treatment of stress urinary incontinence. Although no significant differences

were shown in stress urinary incontinence treatment among both groups, commercial decellularized human fascia lata was associated with a higher recurrence rate [181].

Porcine decellularized pericardium was combined with a rapid-degrading porous collagen matrix and loaded with mice pancreatic islets for beta cell replacement therapy in type 1 diabetes. Scaffolds were transplanted into the epididymal fat pad in a syngeneic mouse model and demonstrated rapid reversal of hyperglycemia with optimal vascularization and integration of islets within the fat tissue [41].

Beyond cardiovascular, musculoskeletal, skin, fetal membranes, ocular, and *in vitro* applications, there are several more different clinical applications where decellularized inner body membranes may be applied. In this section, studies regarding periodontal regeneration, pharyngocutaneous fistulas and stress urinary incontinence treatment, and beta cell replacement therapy in type 1 diabetes were highlighted.

I-5. FINAL REMARKS

Decellularized inner body membranes are promising tools for tissue engineering applications and have been studied for a variety of clinical purposes as previously described. Decellularized inner body membranes have been studied for the regeneration of myocardium, bone, cartilage, tendon, fetal membranes, skin, and cornea. Moreover, these membranes were used for the creation of heart valve substitutes, vascular grafts and *in vitro* models. Standardized decellularization protocols of inner body membranes for specific applications are needed in order to easily compare results and outcomes, and to scale up the production of these membranes for their broad utilization in clinics. Several works crosslink or combine decellularized inner body membranes with synthetic biomaterials to improve their mechanical properties and reduce their biodegradability *in vivo*. Nevertheless, few works compare these different processing methods for the same application, preventing the understanding of its clinical importance.

The availability of the inner body membranes is an important issue. Normally, xenogeneic membranes are used to avoid two surgeries (one for harvesting the material and another to implant the processed material). Another solution is the use of human cadaveric inner body membranes. Regarding placental membranes, this problem does not arise, since placentas are considered a biological waste and are available after full-term pregnancies. This explains the wide interest and study of decellularized amniotic membrane for tissue engineering and regenerative medicine applications.

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SECTION 2

EXPERIMENTAL DESIGN

Chapter II

Materials & Methods

Chapter II

Materials and Methods

OVERVIEW

This chapter provides a detailed description of the materials, methodologies and procedures used to develop the work reported in chapters III to VII of this thesis, complementing the information present in the “Materials and Methods” subsections of those chapters.

Firstly, a description of the materials used, namely human chorion membrane and polycaprolactone is presented. In the second part, the methodologies are described providing more details on its fundamentals and the rationale that lead to its use. The production techniques, such as decellularization and electrospinning are described as well as the methodology used for the characterization of the decellularized human chorion membrane (dHCM). Moreover, the *in vitro* and *in vivo* assays performed to test and validate the developed systems will be presented and justified.

In summary, this chapter aims to support the methodologies selected and used in chapters III to VI to answer the specific hypothesis and research questions studied.

II-1. MATERIALS

II-1.1. Human chorion membrane (HCM)

The human placenta is composed by two fetal membranes, the amniotic membrane and the chorion membrane. The HCM is attached to the amnion membrane in the maternal side [1] and it is composed by three different layers: 1) the reticular layer that contacts with the spongy layer of the amniotic membrane and is composed by collagen type I, III, IV, V and VI; 2) the basement membrane that anchors the reticular and trophoblast layers and is composed by collagen type IV, fibronectin and laminin; and 3) the trophoblast layer that contacts with the maternal decidua and is composed by 2-10 layers of trophoblasts [1,2].

HCM were obtained from human placentas collected from elective and term cesarean sections to limit microorganism's contamination associated with vaginal delivery. An informed consent was signed by all donors and the collection of human placentas followed a protocol approved by the ethical committee of the Hospital de Braga (where cesarean sections were performed) and of the University of Minho (CESH 030/2016; SECVS 136/2015, respectively). Sterile conditions were maintained during all processing steps.

After cesarean section, human placentas were stored in Dupleco's phosphate buffered saline (D-PBS; #21600-044, Alfacene) with 10% antibiotic/antimycotic (#15240062, Thermo Fisher Scientific) at 4 °C for a maximum of one day. The HCM was obtained from the less vascular part of the human placenta, i.e., the chorionic plate was discarded. The amniotic membrane was separated from the HCM by gently pushing. Then, the HCM was washed with sterile phosphate buffered saline (PBS; #P4417, Sigma-Aldrich) once to remove the blood excess and stored at -80 °C until further use.

In this thesis, the decellularization process of the HCM was optimized and the decellularized human chorion membrane (dHCM) was characterized and studied for different tissue engineering and regenerative medicine applications.

II-1.2. Polycaprolactone (PCL)

PCL is a biodegradable polymer widely used in tissue engineering and regenerative medicine applications, for long-term implantable devices, such as drug delivery systems, sutures and scaffolds [3].

Moreover, due to its biocompatibility and ability to form blends and copolymers with other polymers, it has been approved by Food and Drug Administration (FDA) for applications in the human body [3].

PCL is prepared by ring opening polymerization of ϵ -caprolactone, it has a melting point around 60 °C, and presents excellent solubility in common organic solvents. For these reasons, PCL (Mw 80,000, #440744, Sigma-Aldrich) was used on chapter VII of this thesis to produce nanofiber meshes.

II-2. METHODS

II-2.1. Production Techniques

Two main productions techniques were used in this thesis: decellularization of the HCM and electrospinning to produce nanofiber meshes of PCL.

II-2.1.1. Decellularization

The decellularization process is characterized by cell removal while maintaining the tissue characteristics [4]. In this thesis, the optimized decellularization method of HCM is described in chapter III and comprised a combination of mechanical, detergent and enzymatic techniques. Different pools of various placentas were used in order to ensure the traceability of the results.

II-2.1.2. Electrospinning

The electrospinning technique is based on the electrohydrodynamic phenomena occurring when a sufficiently high voltage is applied to a liquid droplet, a charge accumulates at the liquid surface. So, when the electrostatic repulsion is higher than the surface tension, the liquid meniscus is deformed into a conically shaped structure (the Taylor cone) and a charged liquid jet is formed and ejected towards a collector. Depending on the solution viscosity, solid fibers will be formed as the solvent evaporates, resulting in a fiber mat deposited on the collector [5].

On chapter VII, electrospinning was used to create PCL electrospun meshes (PCLem). When fibers are produced, they are deposited in the collector. Fibers stack one over another building several interconnections due to the presence of residual solvent. This nanofiber organization resembles the

extracellular matrix (ECM) [5]. So, PCLem were used as a synthetic homologous of dHCM for the treatment of congenital diaphragmatic hernias.

A solution of 20% PCL was prepared with a solvent mixture of chloroform (#C/4960/17, ENZYMATIC) and dimethylformamide (#444923, Laborspirit) at 8:2 ratio. The PCL solution was electrospun using a 5 mL syringe (B-Braun), a metallic needle with 0.8 cm of external tip diameter and by applying a voltage of 15.5 kV. The needle tip to ground collector distance was 20 cm and the flow rate of 2 mL/h. After the complete processing of 1 mL of PCL solution (30 min), PCLem were allowed to air dry for 1 day.

II-2.2. Decellularized human chorion membrane (dHCM) characterization

After decellularization it is important to evaluate the efficacy of this process by characterizing the tissue regarding its DNA content, protein composition and mechanical properties.

II-2.2.1. Absence of nuclei, DNA extraction and quantification

To avoid the immunogenic reaction caused by cells, the decellularized tissue should have no visible nuclei, and any remaining DNA fragments should have with less than 200 bp and less than 50 ng of dsDNA/mg of dry tissue [4]. To verify the presence of nuclei in decellularized tissue, samples were fixed with 10% formalin and embedded in paraffin. Transverse sections with 5 μ m were cut using a microtome and hematoxylin and eosin (H&E) and 4',6'-diamino-2-fenil-indol (DAPI) staining were performed. H&E is one of the most common histology stains, staining cell nuclei in blue (hematoxylin) and extracellular matrix and cytoplasm in pink (eosin) [6] while DAPI is a fluorescent stain that binds to the adenine-thymine-rich regions of DNA [7]. For H&E staining samples were stained with hematoxylin (#7212, Thermo Fisher Scientific) for 1 min, washed for 30 s and stained with eosin for 10 min (#71204, Thermo Fisher Scientific). Then, slides were washed with water, let to dry and rinsed with alcohol, cleared in xylene and mounted in Entellan rapid (#107960, Merck). Slides were observed under an optical microscope with a coupled digital camera (DM750, Leica). For DAPI staining, samples were incubated with DAPI (#40009, VWR) at 1:10000 for 1-5 min, washed and analyzed using a fluorescence microscope with a coupled camera (Axio Imager Z1m, Zeiss) and processed using Zeiss Zen software. For the DNA extraction, human chorion membranes (native and decellularized) were air dried and weighted. The total DNA was extracted using DNeasy Blood and Tissue Kit (#69504, Qiagen) according to manufacturer's

instructions. The quantification of dsDNA was performed using the Quant-iT PicoGreen dsDNA Assay Kit (#P7589, Invitrogen) according to manufacturer's instructions. To assess the size of DNA fragments, electrophoresis was performed using a 1% agarose gel and GeneRuler DNA Laddered Mix (#SM0334, Thermo Fisher Scientific).

II-2.2.2. Scanning electron microscopy (SEM)

SEM is a type of electron microscopy where the sample is scanned using high energy electrons. The electrons interact with atoms in the sample and the reflected electrons/X-rays are analyzed, giving information about the sample's surface topography and composition. When analyzing a non-conductive material, the specimen has to be coated with a conductive layer of metal or carbon [8]. In this thesis, SEM was used in Chapter IV and VI to analyze dHCM topography and platelets and red blood cells morphology, respectively. Samples were fixed with 2.5% of glutaraldehyde (#G5882, Sigma-Aldrich) in PBS. After three washes with PBS, samples were dehydrated with increasing concentrations of ethanol (10% - 100%) and treated with hexamethyldisilazane (HMD) (#440191, Sigma-Aldrich). Then, samples were air-dried, mounted in SEM pins using carbon tape, and coated with gold using a Sputter Coater (#EM ACE600, Leica). Micrographs were collected with a scanning electron microscope with energy dispersive spectroscopy (EDS) (#JSM-6010 LV, JEOL) in chapter IV and with a high-resolution field emission scanning electron microscope with focused ion beam (AURIGA COMPACT, Zeiss) in chapter VI.

II-2.2.3. Swelling assay

The swelling behavior of a material influences its stability in aqueous medium [9]. In chapter IV, the swelling behavior of dHCM was studied in order to characterize its stability during cell culture and storage. Dry samples were weighted and immersed in D-PBS or culture medium. At different time-points (0.5 h, 1.5 h, 2.5 h, 3.5 h, 4.5 h, and 5.5 h), the excess of liquid was removed (using filter paper) and samples were weighted. After each time-point, samples were re-immersed in liquid until the next time-point. The process was repeated until the equilibrium (stable percentage of water uptake) was reached.

II-2.2.4. Collagen and sulfated glycosaminoglycans (GAGs) quantification

The maintenance of collagen and sulfated GAGs in a decellularized tissue is important since these components are important to maintain the integrity and function of the tissue [10]. Nevertheless, the decellularization process is associated with collagen damage and disruption and with GAGs removal, especially when detergents such as SDS and Triton-X are used [4]. So, during decellularization, it is important to maintain a good balance between ECM preservation and complete cellular removal. In chapter IV, collagen and sulfated GAGs were quantified to characterize the dHCM. Sircol Collagen Assay Kit (#S5000, Biocolor) and Sircol Insoluble Collagen Assay Kit (#2000, Bicolor) were used (in accordance with manufacturer's instructions) to extract and quantify the content of soluble and insoluble collagen (respectively) of HCM, dHCM and trophoblast layer. Sulfated GAGs content from HCM, dHCM and trophoblast layer were extracted and quantified as previously described [11]. Briefly, samples were digested using 0.5 mg/mL papain (#P4762, Sigma-Aldrich) and the supernatant was stained with 1,9-dimethylmethylene blue (DMB). Before measurement, samples were diluted 1:10 in distilled water and a dilution series of chondroitin sulfate in distilled water (50 µg/mL) was used as standard solution. Then, using a microplate reader (Synergy HT, Biotek), the absorbance of the mixture of 20 µL of diluted samples and standards with 250 µL of DMB (#341088, Sigma-Aldrich) was read at 525 nm.

II-2.2.5. Protein characterization

To characterize a decellularized tissue it is important to study its protein composition and distribution. In Chapter IV, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to demonstrate the reproducibility of the decellularized protocol regarding protein composition, Dot-Blot was used to identify some ECM proteins and protein immunolocalization allowed the study of protein distribution among the tissue. For SDS-PAGE and Dot Blot techniques, protein from HCM and dHCM was extracted using Tissue Extraction Reagent I (#FNN0071, Thermo Fisher Scientific) with a protease inhibitor cocktail (#P8340, Sigma-Aldrich). Then, the soluble protein content was determined using Protein Assay Dye Reagent Concentrate (#5000006, Biorad), according to the manufacturer's instructions.

SDS-PAGE is a common method used to separate proteins according to its molecular weight, since the combination of SDS with polyacrylamide gel allows for the elimination of both the structure and charge influence [12]. Herein, a 4% stacking gel and 9% running gel were prepared using SDS Gel

Preparation Kit (#08091, Sigma-Aldrich). For each sample, 0.5 µg/µL of protein was loaded in the respective well. Following SDS-PAGE, the gel was stained with Coomassie Blue R-250 (HS-604, National Diagnostics) and an image was obtained using a Transilluminator (Biospectrum ACCHEMI HR 410).

Dot blot is a simple technique used to detect proteins. It may be characterized as a simplification of the western blot method, where proteins to be detected do not have to be firstly separated by electrophoresis [13,14]. One drop of soluble protein from each sample was placed in a nitrocellulose membrane. After drying, membranes were blocked for 1 h with 5% BSA (#A2153, Laborspirit) with agitation at room temperature (RT). Membranes were then incubated with primary antibodies overnight: mouse anticollagen type I 1:1000 (#ab90395, abcam); rabbit anticollagen type IV, 1:500 (#ab6311, abcam); rabbit antifibronectin 1:500 (#ab45688, abcam); and mouse antilaminin, 1:500 (#L8271, Sigma-Aldrich). After 3 washes for 5 min with Tris Buffered Saline (TBS) with tween 20 (#P1379, Laborspirit), R.T.U. VECTASTAIN® Universal ABC Elite® Kit (#PK-7200, Vector Laboratories) was used as a secondary antibody, in accordance with manufacturer's instructions. Revelation was performed using Peroxidase Substrate Kit (DAB) (#SK-4100, Vector Laboratories). Extraction buffer was used as a negative control while collagen type I (#sc-136157, Santa Cruz Biotechnology), collagen type IV (#C5533, Sigma-Aldrich), fibronectin (#FC010, Sigma-Aldrich) and laminin (#L6274, Sigma-Aldrich) were all used as positive controls.

Immunolocalization of proteins was performed in paraffin embedded samples sectioned at 5 µm. Samples were incubated with mouse anticollagen type I 1:100 (#ab90395, abcam); rabbit anticollagen type IV, 1:50 (#ab6311, abcam); rabbit antifibronectin 1:300 (#ab45688, abcam); and mouse antilaminin, 1:300 (#L8271, Sigma-Aldrich), overnight at 4 °C in a humidified atmosphere. Then, R.T.U. VECTASTAIN® Universal ABC Elite® Kit (#PK-7200, Vector Laboratories) was used as secondary antibody and the signal was revealed using DAB (#SK-4100, Vector Laboratories). Samples were counterstained with hematoxylin, mounted in an aqueous mounting medium, and observed under an optical microscope with a coupled camera (DM750, Leica).

II-2.2.6. Mechanical properties

To study of the biomaterial's mechanical properties is of great importance, since it allows to predict the material behavior under stress [15]. In chapter IV, dHCM stiffness (Young's modulus) and tensile strength were studied. HCM and dHCM dry samples were cut and placed in a universal mechanical testing equipment (#5543, INSTRON) equipped with a 1 kN load cell and were hydrated. The strain rate

was defined at 5 mm/min and a 10 mm gauge length was used in the tensile tests. Tests were finished when the specimens break.

II-2.3. *In vitro* assays

Materials used in tissue engineering and regenerative medicine applications need to be tested for their cytotoxicity. An easy and cost-effective way to do that is by culturing cells onto the materials *in vitro*. Moreover, the maintenance of cell functionality is important while seeded into materials. So, cell proliferation, morphology and overall performance need to be evaluated.

II-2.3.1. Cells

Over the past 50 years there has been an increase in the use of cells as models, substrates and tools for basic research and industrial applications. Cell lines are defined as cells that have been continually passaged over a long period of time, maintaining homogeneous genotypic and phenotypic characteristics. So, cell lines are widely used in several *in vitro* studies, since they are, generally well characterized, easy to handle and facilitate results comparisons between groups. Nevertheless, cell lines have lost some characteristics of the original tissue from which they were isolated, not mimicking, very closely, the *in vivo* environment. Thus, primary cells may be used. Although primary cells are associated with a limited lifespan, they allow the study of donor variability and tissue complexity.

II-2.3.1.1 Human umbilical vein cell line (EA.hy926)

Human umbilical vein cell line was established by fusing a human umbilical vein endothelial cell with a human carcinoma cell line (A549). The features of this cell line make it a good model system for human vascular endothelial cells [16,17]. EA.hy926 cell line (#ATCC-CCL-2922, LCG Standards) was used in Chapter IV of this thesis to assess the biocompatibility of dHCM. This cell line was cultured using Duplecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS (#A3160801, Thermo Fisher Scientific) and 1% penicillin/streptomycin (#15240062, Thermo Fisher Scientific) in T150 flasks at 37°C in a humidified 5% CO₂ atmosphere. When 80% confluence was reached, cells were trypsinized using using TripLE express (#12605028, Alfacene) for 5 min at 37 °C and centrifuge at 300 *g* for 5 min (Eppendorf 5810R centrifuge equipped with an A-4-62 rotor).

II-2.3.1.2 Human fetal lung fibroblast cell line (MRC-5)

The MRC-5 cell line was established in 1966 by Jacobs, et al. from the lung tissue of a 14-week-old male fetus aborted from a 27-year-old Caucasian woman. This cell line is composed of human lung fibroblasts known to reach senescence in around 45 population doublings [18]. MRC-5 cell line (#ATCC-CCL-171, LGC Standards) was used in Chapter IV of this thesis as a model of normal cell to study the biocompatibility of dHCM. This cell line was cultured using DMEM supplemented with 10% FBS (#A3160801, Thermo Fisher Scientific) and 1% penicillin/streptomycin (#15240062, Thermo Fisher Scientific) in T150 flasks at 37°C in a humidified 5% CO₂ atmosphere. After reaching 80% of confluence, cells were trypsinized using TripLE express (#12605028, Alfacene) for 5 min at 37 °C and centrifuge at 300 *g* for 5 min (Eppendorf 5810R centrifuge equipped with an A-4-62 rotor).

II-2.3.1.3 Mouse brain capillary endothelial cell line (b.End3)

b.End3 is a mouse brain capillary endothelial cell line derived from BALB/c mice. Mice's brain endothelial cells were isolated and immortalized by the infection of the primary cells with a retrovirus coding for the Polyoma virus middle T-antigen. b.End3 together with b.End5 cell lines are the only immortalized brain endothelial cell lines available from commercial cell banks. b.End3 was shown to functionally express several transporters important to the blood-brain barrier (BBB) penetration of xenobiotics, enabling the study of mechanistic aspects of drug-BBB interactions [19]. This cell line was used in Chapter V to functionally validate a BBB *in vitro* model using dHCM. b.End3 (#ATCC CRL-2299, LGC Standards) were cultured using DMEM high glucose (#D5648, Sigma-Aldrich) with 1.5 mg/mL sodium bicarbonate (#S586, Sigma-Aldrich), 1% sodium pyruvate (#11360039, Gibco), 10 % fetal bovine serum (FBS; #A3160801, Thermo Fisher Scientific) and 1% Penicillin/Streptomycin (#15240062, Thermo Fisher Scientific) in T150 flasks at 37°C in a humidified 5% CO₂ atmosphere. When cells reached 80% confluency, they were trypsinized using TripLE express (#12605028, Alfacene) for 5-10 min at 37 °C and centrifuge at 300 *g* for 5 min (Eppendorf 5810R centrifuge equipped with an A-4-62 rotor).

II-2.3.1.4 Mouse cerebellum astrocyte cell line (C8-D1A)

C8-D1A is a spontaneously immortalized mouse cerebellum astrocyte cell line derived from the cerebellum of 8 days-old C57BL/6 mice [20]. C8-D1A cell present an astrocyte type III phenotype [20]

and were used in Chapter V to functionally validate BBB *in vitro* model using dHCM. Cells (#ATCC CRL-2299, LGC Standards) were cultured in T150 flasks at 37°C in a humidified 5% CO₂ atmosphere using DMEM high glucose (#D5648, Sigma-Aldrich) with 1.5 mg/mL sodium bicarbonate (#S586, Sigma-Aldrich), 1% sodium pyruvate (#11360039, Gibco), 10 % fetal bovine serum (FBS; #A3160801, Thermo Fisher Scientific) and 1% Penicillin/Streptomycin (#15240062, Thermo Fisher Scientific). After reaching 80% confluence, cells were trypsinized using TripLE express (#12605028, AlfaGene) for 1-2 min, without shaking, at 37 °C and centrifuge at 300 *g* for 5 min (Eppendorf 5810R centrifuge equipped with an A-4-62 rotor).

II-2.3.1.5 Human umbilical vein endothelial cells (HUVEC)

HUVECs are primary cells derived from the endothelium of veins from the umbilical cord. HUVECs (#C-003-5C, AlfaGene) were used in Chapter VI to perform the tube formation assay. HUVECs were cultured in medium 200 (#M-200-500, Gibco) supplemented with large vessels endothelial supplement (LVES, #A14608, Gibco) in T75 and T150 flasks at 37°C in a humidifier incubator with 5% CO₂. When 80% confluency was reached cells were trypsinized using TripLE express (#12605028, AlfaGene) for 5 min at 37 °C and centrifuge at 300 *g* for 5 min (Eppendorf 5810R centrifuge equipped with an A-4-62 rotor).

II-2.3.2. Cell seeding

Cell seeding is an important step to achieve a successful colonization and a uniform distribution of the cells into the materials. Cell seeding can be static or dynamic [21]. In this thesis, static cell seeding was used. Basically, a concentrated cell suspension was pipetted into the surfaces.

dHCM were always mounted in cell crown inserts for 24-well plates (#Z742380-12EA, Sigma-Aldrich) with trophoblast layer side or amniotic layer side facing the upper side of the insert and immersed in culture medium overnight before cell seeding. Cell crown inserts were important to allow the stretching and immobilization of dHCM during the assays. Depending on the assay, Polyethylene terephthalate (PET) 0.4 μ m for 24-well plates (#MCHT24H48, Millipore, Burlington, MA, USA) or bottom of the well coated with Matrigel® (#354234, Enzifarma) were used as control. At least, three independent assays for each cell line were performed.

II-2.3.2.1 Cell-culture plastic surfaces

In chapter VI HUVECs were seeded in the bottom of the well of 48-well plates coated with 50 μL cm^2 Matrigel® (#354234, Enzifarma) and used as positive controls for tube assay formation. HUVECs were seeded in 300 μL of culture medium with a density of 25×10^3 cells/ cm^2 .

II-2.3.2.2 Inserts

As mentioned before, inserts (both dHCM mounted in cell crown inserts and millicell hanging cell culture inserts) were immersed in culture medium overnight before seeding. Then, the culture medium was removed and for dHCM mounted inserts, 900 μL of culture medium were added at the bottom side of the insert and for millicell hanging cell culture inserts, 1300 μL were added at the bottom side of the insert. The different culture medium volumes are related with the different areas that the inserts fill in the well and the different surface areas. dHCM mounted inserts have a surface area of 0.99 cm^2 while Millicell hanging cell culture inserts have 0.33 cm^2 . So, the volumes were added at the bottom side of the inserts, were defined to allow the same proportion (mL/cm^2) of culture medium in the upper side of the insert (600 μL in dHCM mounted inserts and 200 μL in Millicell hanging cell culture inserts) and to have the same final volume of culture medium in the well (1500 μL).

II..2.3.2.2.1 Monoculture

Monocultures of EA.hy926, MRC-5, b.End3, and C8-D1A cell lines were performed in chapters IV and V to verify dHCM cytocompatibility. For these studies, cell seeding on both inserts (dHCM mounted inserts and Millicell hanging cell culture inserts) was done by dropping a 100 μL suspension containing 20×10^3 cells/ cm^2 . After an incubation period of 3 h to allow cells to adhere to the surfaces, 500 μL of culture medium was added to the upper side of dHCM mounted inserts and 100 μL of culture medium was added to the upper side of Millicell hanging cell culture inserts.

In chapter VI, a monoculture of HUVECs was used to evaluate its performance in a tube formation assay. Cells were seeded in 300 μL of culture medium with a density of 25×10^3 cells/ cm^2 . Due to the nature of the tube formation assay, no further culture medium was added, and the first time-point was measured 4 h after seeding.

II.2.3.2.2.2 Co-culture

A co-culture system was developed in chapter V using b.End3 and C8-D1A cell lines to create a BBB model. Cells were seeded at the same time with a density of 20×10^3 cells/cm² in 100 μ L of culture medium. b.End3 cells were seeded in the upper side of the dHCM mounted inserts, while C8-D1A cells were seeded in the bottom of the well. After 3 h, 800 μ L of culture medium were added to the wells seeded with C8-D1A cells. Then, dHCM mounted inserts seeded with b.End3 cells were moved to the wells seeded with C8-D1A cells. Finally, 500 μ L of culture medium were added to the upper side of the insert.

II-2.3.3. Metabolic activity, cell viability and functionality

The cytotoxicity effects of a material may be evaluated by cell death and for alterations in its morphology and are normally associated with materials' topography and/or exposure to molecules or compounds present or released during the culture time. Thus, when seeding cells into a material it is important to study cellular viability, morphology, distribution and functionality [22].

II-2.3.3.1 MTS

The evaluation of cells' metabolic activity is a commonly used parameter to assess the cytotoxicity of a material or compound. There are several alternative quantitative assays to determine cells' metabolic activity, such as MTT, MTS and alamarBlue® [22]. In this thesis, MTS was used in chapters IV and V to study the cytocompatibility of dHCM using EA.hy926, MRC-5, b.End3, and C8-D1A cell lines.

MTS assay is based on the reduction of tetrazolium compound (MTS) into colored formazan by cells in culture. This conversion is performed by dehydrogenase enzymes (presumably NADH or NADPH) present in metabolically active cells that will transfer electrons to MTS, resulting in a reduced brown formazan, soluble in culture medium and directly proportional to the number of viable cells [22,23]. In this thesis, the CellTiter 96 AQueous One Solution cell proliferation assay (#G3581, Promega) was diluted in culture medium (1:5) and added to each well and incubated for 3 h, at 37 °C in a humidified 5% CO₂ atmosphere. Then, the absorbance of the MTS reaction medium of each well was read in triplicate at 490 nm using a microplate reader (Synergy HT, Bio-TEK).

II-2.3.3.2 Tube formation

The tube formation assay is a well-established, rapid and quantitative method for determining the angiogenic activity of materials and/or compounds. This assay was first described in 1988 and is based on the principle that endothelial cells retain the ability to divide and migrate rapidly in response to angiogenic signals. Thus, in the presence of angiogenic signals or when seeded on a matrix of basement membrane extracts, endothelial cells will be induced to differentiate and form tube-like structures characterized by a lumen surrounded by endothelial cells linked through junctional complexes. In general, tube formation occurs within 2-6 h after seeding or exposure to a stimulus. Nevertheless, it is dependent of the quantity and type of angiogenic stimuli [24].

In chapter VI a tube formation assay was performed using HUVECs to study the angiogenic potential of dHCM. The bottom of the well coated with Matrigel® (#354234, Enzifarma) was used as positive control, since the capacity of Matrigel® to induce endothelial cells to form tubular-like structures is well characterized [24]. Briefly, the bottom of 48 well-plates (surface area of 0.95 cm²) was coated with 47.5 µL of Matrigel® and left to solidify for 1 h at 37 °C. As previously described, dHCM were mounted in cell culture inserts and immersed in culture medium overnight (surface area of 0.99 cm²). HUVECs were seeded at a density of 25x10³ cells/cm² in 300 µL of culture medium. At different time points (4, 8, 12 h) the culture medium was collected, and cells were fixed with 10% formalin (#5701, Thermo Fisher Scientific) to further quantify the released angiogenic growth factors and to analyze its tubular-like structures, respectively.

II-2.3.3.3 Cell morphology, distribution and functionality

There are several ways to study cell morphology and distribution. Due to the nature of dHCM, Phalloidin/DAPI staining has demonstrated to be the most adequate method. In chapter V, phalloidin/DAPI staining was used to analyze the morphology and distribution of b.End3 and C8-D1A cell lines when seeded at the surface of the dHCM. Briefly, at specific time points (day 3 and 7 of culture) cells were fixed using 10% formalin (#5701, Thermo Fisher Scientific) for, at least, 24 h at RT. Samples were incubated with 1:200 Phalloidin-TRITC (#P1951, Sigma-Aldrich) at RT for 1 h and counterstained with DAPI (#40009, VWR) at 1:10000 for 1-5 min. For PET membranes (Millicell hanging cell culture inserts), DAPI was used at 1:1000. On chapter VI, the platelet distribution on the surfaces was also evaluated using the same protocol for phalloidin staining.

Cell functionality was assessed by immunofluorescence and phalloidin staining in chapters V and VI, respectively. In chapter V, the expression of receptors important for BBB *in vitro* model functionality by b.End3 cells, such as glucose transporter 1 (GLUT-1) and transferrin receptor, was studied. In summary, after a permeabilization with 0.5% Triton X-100 (#A16046-0100, Alfa Aesar) for 10 min at RT and a blockage with 5% BSA (#A2153, Sigma-Aldrich) for 1 h at RT, samples were incubated with rabbit anti-GLUT1 1:50 (#ab15309, abcam), and rabbit anti-Transferrin Receptor 5 µg/mL (#ab84036, abcam) antibodies, overnight at 4 °C. Donkey anti-rabbit 594 1:500 (#A21207, alfa gene), used as secondary antibody, was incubated for 1 h at RT. Then, samples were counterstained with DAPI (#40009, VWR) at 1:10000. On chapter VI, the HUVECs ability of forming tube-like structures was also analyzed by phalloidin staining, as described above.

A fluorescence microscope with a coupled camera (Axio Imager Z1m, Zeiss) was used to obtain images that were further analyzed with the Zeiss Zen microscope software.

II-2.3.4. Transendothelial electrical resistance (TEER)

TEER is a measure of barrier tightness, characterizing the impedance across an endothelium layer. A higher TEER is usually associated with stronger tight junctions and consequently with an enhanced resistance to paracellular diffusion. TEER measurement is a commonly criteria to evaluate the quality of BBB *in vitro* models, especially transwell models [25]. In chapter V, TEER was measured using a Millicell® ERS-2 Electrical System (MERS00002, Millipore) in accordance with manufacturer's instructions. Each condition was performed in triplicate at three different time-points (3 and 7 days), and five TEER measurements were obtained per sample. At least three independent assays were performed for each condition.

II-2.3.5. Permeability assays

The study of membrane permeability was further evaluated in combination with TEER measurements [25]. In chapter V, three different transportation mechanisms across the BBB were tested: membrane diffusion (70 kDa FITC-dextran), solute carrier (caffeine), and receptor-mediated (glucose). Permeability assays on transwell models are based on the principle that molecules added in the upper chamber of the transwell insert, will naturally pass to the bottom chamber. So, it is possible to study the influence of a cell monolayer modulating the permeability of the membrane.

At different time-points (3 and 7 days) the inserts were transferred to a new receiver 24-well plate with 1 mL of fresh culture medium. Then, 400 μ L of working solution were added to the upper side of the insert. For membrane diffusion 10 μ g/mL of 4 kDa or 70 kDa FITC-dextran (#46944 and #46945, Sigma-Aldrich, respectively) in culture medium were added and the inserts were incubated for 2 h at 37 °C in a humidifier incubator with 5% CO₂. Then, 100 μ L of culture medium were collected from the receiver plate and transferred to an opaque 96-well plate where fluorescence was read using an excitation of 485 nm and emission of 535 nm, in a microplate reader (SynergyHT, Biotek). For solute carrier and receptor-mediated transportation, 75 μ g/mL of Caffeine (#C0750, Sigma-Aldrich) and 4.41 mg/mL of glucose (#G8270, Sigma-Aldrich) in culture medium (respectively), were used as working solutions. After a 3 h incubation, Caffeine ELISA kit (#DEIA6842, Creative Diagnostics) and Glucose Assay Kit (#ab102517, abcam) were used to quantify caffeine and glucose in the receiver plate, respectively. The permeability coefficient was calculated according to the following equation:

$$\text{Permeability Coefficient} = (V_{UP} / (S_A * t)) * (C_R * C_{UP})$$

Where: V_{UP} is the volume added to the upper side of the insert (400 μ L); S_A is the surface area of the insert (0.95 cm²); t is the time in s; C_R is the concentration in the receiver plate; and C_{UP} is the initial concentration added to the upper side of the insert.

II-2.3.6. Enzyme-linked immunosorbent assay (ELISA)

ELISA represents a simple and sensitive assay to quantify specific antigens, such as peptides, proteins, antibodies, and hormones. ELISA is an immunoassay relying on the specific binding between the antibody and the target antigen. Moreover, it is associated with a detection system that allows the quantification of the bound antigen. Generally, a specific primary antibody is attached to the surface of a 96-well plate that will bind to the antigen. Then, a secondary antibody, an enzyme and a substrate that links the enzyme are added. The color change can be measured using a spectrometer. The antigen concentration is determined using a standard calibration curve [26].

ELISA was used in chapters V and VI of this thesis. In chapter V, ELISA was used in permeability assays to assess the functionality of the developed BBB model using dHCM. Basically, after the permeability assay, ELISA was used to quantify the tested molecules present in the collected medium. ELISA kits for caffeine (#DEIA6842, Creative Diagnostics), glucose (#ab102517, abcam), and cholesterol (#STA-384, Cell Biolabs, Inc.) were used in accordance with manufacturer's instructions. On chapter VI,

ELISA was used as a complement to the tube assay formation for the quantification of angiopoietin 2 (#ab99971, abcam), an angiogenic growth factor secreted by endothelial cells.

II-2.3.7. Transferrin uptake assay

Transferrin receptor has been exploiting for the delivery of protein therapies to the brain [27]. So, on chapter V the transferrin uptake was evaluated to validate the functionality of the BBB *in vitro* model. Firstly, cells were starved for 1 h using culture medium without FBS. Then, the culture medium in the upper chamber of the insert was removed and replaced by 400 μ L of a working solution of 25 μ g/mL of biotinylated transferrin (#T3915, Sigma-Aldrich) in starving medium. Cells were incubated for 30 min and fixed with 10% formalin. After three washes with PBS, biotin signal was revealed using streptavidin 1:500 (#S11227, Thermo Fisher Scientific). Samples were analyzed under a fluorescence microscope with a coupled camera (Axio Imager Z1m, Zeiss). Images were analyzed with Zeiss Zen microscope software.

II-2.3.8. Hemocompatibility assays

Hemocompatibility is an important criterion that limits the application of blood-contacting biomaterials. When a biomaterial comes in close contact with blood, it should not adversely interact with any blood components and activate or compromise the bioactivity of any blood component [28].

In vitro blood compatibility assays allow for the analysis of a material's hemocompatibility under controllable conditions, enabling direct comparison of outcomes. Although *in vitro* assays eliminate disturbing factors related to flow obstruction, surgery, and other tissue effects, they are associated with a more intense blood contact, since the generated products are not cleared [28]. In chapter VI, static and agitated *in vitro* hemocompatibility assays were performed. Glass surfaces, which show poor hemocompatibility [28] were used as positive controls of cytotoxicity. Fresh blood was collected via venipuncture from healthy human volunteers in BD Vacutainer tubes (#367704, BD Diagnostics – Prenalytical Systems) containing sodium citrate. Platelet rich plasma (PRP), used in protein absorption assay, was provided by Centro Hospitalar de São João (Porto, Portugal) under an approved institutional board protocol (hospital ethical commission approval number 363/18).

II-2.3.8.1 Protein adsorption assay

When a material is in contact with blood, a protein layer mainly composed by plasma proteins (such as fibrinogen, immunoglobulins, fibronectin, vitronectin, factor XI and XII, von Willebrand factor, and plasma prekallikrein) is adsorbed on the material's surface. This event may trigger adverse reactions, such as the activation of coagulation via intrinsic pathway, the activation of leukocytes, which results in inflammation, and the adhesion and activation of platelets that may lead to thrombus formation [28]. For protein adsorption assay, PRP was centrifuged at 800 *g* for 20 min at RT in order to obtain platelet poor plasma (PPP). 300 μ L of PPP or PBS (negative control) were added to both sides of dHCM and glass surfaces. After a 1 h incubation at 37°C, samples were washed three times with PBS. Then, samples were immersed in 1% SDS under agitation for 1 h and sonicated for 20 min. For protein quantification Micro BCA™ Assay Kit (#23235, Thermo Scientific) was used according to manufacturer's instructions.

II-2.3.8.2 Platelet adhesion and aggregation assay

Blood platelets are the smallest (1-3 μ m) and the second most abundant cell type in the blood. In physiological conditions, platelets circulate in the blood in a quiescent state for 7-10 days. However, the exposure of a biomaterial to the blood results in protein adsorption that may lead to an undesired activation of platelets and consequently lead to thrombus formation [28].

Platelet isolation was performed by centrifuge the fresh blood at 200 *g* for 20 min at RT to obtain PRP. All the centrifugation steps of the protocol were performed with no brake applied. The PRP was then mixed with HEP buffer 1:1 ratio (v/v) with 1 μ M prostaglandin E1 (P5515, Sigma-Aldrich) and centrifuged at 100 *g* for 18 min at RT. The supernatant was transferred to a new plastic tube and centrifuged at 800 *g* for 18 min. The pellet was washed twice with Platelet Wash Buffer (without resuspension) and the platelet concentrate was resuspended in 20 mL of Tyrode's buffer containing 5 mM glucose (#G8270, Sigma-Aldrich) and 3% BSA (#A2153, Sigma-Aldrich). Finally, 300 μ L of platelet suspension were seeded in both sides of dHCM and in glass surfaces and incubated for 1 h at 37°C in a humidifier incubator with 5% CO₂.

After the incubation period, samples were washed 3 times with PBS to remove any non-adherent platelets and fixed with 10% formalin or 2.5% glutaraldehyde for fluorescent imaging and SEM, respectively (as described in sections II-2.3.3.3 and II-2.2.2, respectively). Moreover, samples were also immersed in

2% Triton X-100 (#A16046, Thermo Fisher Scientific) in PBS to be used in lactate dehydrogenase (LDH) assay.

Platelets have a high LDH activity. So, platelet adhesion was quantified by measuring LDH released when adherent platelets were lysed using 2% Triton X-100 using the CyQUANT™ LDH Cytotoxicity Assay (#C20301, Invitrogen) according to manufacturer's instructions. The percentage of platelet adhesion on the different materials was determined from a calibration curve obtained by using different dilutions of the seeded platelets.

II-2.3.8.3 Hemolytic assay

Erythrocytes or red blood cells (RBC) are the most abundant cells in the blood and are important for the transport of oxygen from the lung to all tissues and of carbon dioxide from tissues to the lung and its damage may lead to a reduced oxygen transport. Moreover, when RBC are ruptured, they release hemoglobin that can induce toxicity or alter the kidney function. One way to assess the material compatibility with RBC is by the quantification of free hemoglobin, since an increased concentration of free hemoglobin is an indicator of erythrocyte disruption [28].

RBC were isolated by centrifugation of the fresh blood at 200 *g* for 20 min at RT. PRP was used for platelet isolation (as described in subsection II.2.3.8.2), buffy coat was discarded, and RBC (the downer portion) were washed 3 times with PBS. After each wash a centrifugation of 5 min at 3700 rpm was performed. Finally, RBC concentrate was diluted to a final concentration of 5 vol. % and 300 μ L of RBC suspension were seeded in both sides of dHCM. For negative and positive controls, PBS (0 % lysis) and 1 % SDS (82 % lysis) were added to the RBC suspension, respectively. After 1 h incubation at 37°C in a shaking incubator chamber, samples were washed with PBS (3x) and fixed with 2.5% glutaraldehyde (for SEM analysis as described in section II-2.2.2) or centrifuged at 120 *g* for 10 min to further hemoglobin quantification. Supernatants (50 μ L) were transferred into a 96-well plate and the absorbance was measured at 540 nm using a microplate reader (Synergy HT, Biotek). To determine the hemolytic ratio the follow equation was used:

$$\text{Hemolytic ratio} = (\text{Sample} - \text{Negative control}) / (\text{Positive control} - \text{Negative control})$$

Hemolysis ratio was interpreted as follow: < 2% non-hemolytic, 2-5% slightly hemolytic, and > 5% hemolytic samples.

II-2.4. *In vivo* assays

In vivo assays are important to assess material biocompatibility and functionality when implanted. Although *in vitro* models are useful and cost-effective in a primary phase of material characterization, they have limited capacity to recreate the complex *in vivo* environment. Thus, *in vivo* models are needed to understand host response to a biomaterial and are useful to predict its clinical behavior, functionality, safety and biocompatibility [29–31].

Laboratory animal protection legislation assumes that in, specific conditions, it is morally acceptable to use animals for scientific purposes when the following objectives are met: use the minimum number of animals, define legitimate purposes for which laboratory animals may be used, ensure the training ability of all laboratory personnel and researchers, avoid animals use when there are practicably available alternatives, avoid unnecessary pain or distress to animals, provide for the inspection of facilities and procedures, and ensure public responsibility [32].

The choice of an appropriate animal model is intrinsically related to the specific goals of the experiment. In this thesis, dHCM was implanted subcutaneously in mice to assess its biocompatibility (chapter IV). To verify dHCM angiogenesis, a chick chorioallantoic membrane (CAM) assay was used (chapter VI). Moreover, an orthotopic model in pigs was used to study the ability of dHCM to close diaphragmatic hernias (chapter VII).

All studies were performed following EU Directive 2010/63/EU and approved by the animals' ethics committee of the institution where the study was performed. Moreover, all personnel involved in the procedures were approved as competent for animal experimentation by DGAV (Portuguese general directorate of food and veterinary).

II-2.4.1. Subcutaneous implantation in mice

In early phases of research, ectopic models are preferred to orthotopic models because it is easier to identify the tissue response, to compare results with others in the literature, and to obtain the technical skills needed to perform them. Ectopic models are frequently used to assess the foreign-body reaction of a material *in vivo*, since they are able to provide information about chronic inflammatory response, and integration of the biomaterial within the host tissue [29,30]. One of the most commonly used implantation sites is the subcutaneous implantation.

In chapter IV, a total of 20 BALB/c mice aged 9 weeks old (Charles Rivers, Barcelona, Spain) were used to study dHCM biocompatibility *in vivo*. Animals were housed five per cage in a limited-access rodent facility. Food and water were available *ad libitum* and animals were maintained in a climate-controlled room (22.0 ± 0.5 °C) in 12/12 h light/dark cycles. Before surgery, animals were exposed to handling twice a day. On surgery day, mice were anesthetized by an intraperitoneal injection of Domitor (medetomidine 0.5 mg/kg) and Imalgene (ketamine 75 mg/kg). The dorsum of the animals (implantation area) was shaved, cleaned and sterilized with Betadine® and two skin incisions were made (each 0.5 cm length) in each mouse. dHCM was implanted subcutaneously in each of the pockets and the incision was closed using 5-0 sutures (15 animals, 5 animals for each time-point). Empty defects were used as controls, either in the control group (5 animals, 2 animals for the first two time-points and 1 animal in the last time-point) and in the contralateral side of the pocket containing the material. After the procedure, the anesthesia was reversed with a subcutaneous injection of antisedan and the animals were monitored until fully awake (eating and grooming). After 4, 10 or 28 days, animals were euthanized using pentobarbital injection, and dHCM membranes and empty pockets were explanted (with the surrounding tissues).

Explanted samples were fixed with 10% formalin and embedded in paraffin. Histochemical stains such as H&E and Masson's trichrome (MT) were performed in 5 µm thick transversal sections. H&E staining was performed as described in subsection II.2.2.1. MT staining is commonly used and stains cartilage and collagen fibers in blue/green, muscle fibers in red, nuclei in black, cytoplasm in red/pink, and RBCs in red [33]. For MT staining, slides were submitted to Azure B solution for 5 min, stained with hematoxylin for 5 min and washed in picric ethanol for 5 min. After that, samples were stained in Biebrich Scarlet-Acid Fuchsin for 15 min, submitted to 1% phosphomolybdic acid for 5 min and to Aniline Blue for 4 min. Then, slides were washed with water, let to dry, rinsed with alcohol, cleared in xylene, mounted in Entellan rapid, and observed under an optical microscope with a coupled digital camera.

II-2.4.2. Chick chorioallantoic membrane (CAM) assay

CAM assay represents a rapid, simple and cost-effective *in vivo* test to screen the angiogenic potential of biomaterials, due to the highly vascularized nature of the CAM [34,35]. In chapter VI, white fertilized eggs were incubated for 3 days at 37°C and a window was open into the eggshell to evaluate embryo viability. After 7 days, dHCM were implanted on the CAM forming two groups: dHCM reticular layer side in direct contact with CAM and dHCM trophoblast layer side in direct contact with CAM. Some

eggs were left empty and were used as control. Eggs were then returned to the incubator and at day 17, embryos were sacrificed by freezing at -80 °C for 10 min. After fixation with 4% formalin, ex-ovo images were captured for each condition in both sides of the CAM. At least 7 eggs were analyzed per condition (CTR, dHCM reticular layer side and dHCM trophoblast layer side). The macroscopic evaluation of the angiogenic response was carried out as previously described [36]. Moreover, an automated angiogenesis quantification analysis was performed using the Vessel Analysis plugin with Image J, where the measurements of vascular density and vascular length density are expressed as percentage area.

II-2.4.3. Congenital diaphragmatic hernia (CDH) model in pigs

In chapter VII, dHCM was compared with electropun nanofiber meshes (PCLem) and GoreTex® for congenital diaphragmatic hernia repair in pigs. So, a novel diaphragmatic hernia animal model was established using thoracoscopy in sexually immature farm pigs, allowing for characterization of the effect of subject growth in defect size and regeneration.

Fourteen farm male pigs (*Sus scrofa domestica*) with approximately 6 weeks of age (6.54 ± 0.57 kg body weight) were used. Animals were housed in a facility with controlled temperature (24 ± 2 °C) and automatic air renewal in socially stable groups of 2 or 3 animals in each pen. Dry pellet food was provided once a day, and water was available *ad libitum*. Wood shavings, fresh fruit, ropes and balls were provided for enrichment. Animals were randomly assigned to 3 different experimental groups of 4 animals each, determined by the type of patch used during thoracoscopic diaphragmatic repair (Gore-Tex®, PCLem, or dHCM). Two additional animals were used as age and weight-matched controls and submitted to thoracoscopy without creation of diaphragmatic defect (sham group).

After, at least, 7 days for acclimatization, and at the end of the protocol, animals were submitted to a chest X-Ray evaluation under sedation in order to document lung pathology, hernia recurrence or diaphragmatic eventration, and skeletal deformations such as scoliosis or thoracic wall deformities.

All surgical procedures were performed under general anesthesia with endotracheal intubation and mechanical ventilation. An 8 h fasting period was performed, while water was provided until sedation time. Animals were anesthetized with a combination of ketamine (20 mg/kg, intramuscularly (IM)), xylazine (2 mg/kg), and atropine (0.04 mg/kg, IM), followed by propofol (4 mg/kg, intravenously (IV)) and maintained with continuous propofol infusion (20 mg/kg/h, IV) and buprenorphine (0.05 mg/kg).

All surgical interventions were performed under sterile conditions and continuous cardiorespiratory monitoring. Animals were positioned in right lateral decubitus. A 5 mm port was placed

in the 6th intercostal space aligning with the shoulder joint, for introduction of a 5 mm 30° telescope. Moreover, two working ports were placed in the 7th intercostal space under endoscopic vision, one 3 mm port along a line passing through the tip of the left scapula and the other 5 mm port approximately 5 cm from the left costal arch, allowing access to the diaphragm. Capnothorax was established with an inflation rate of 1 L/min and maintained at a maximum pressure of 3 mmHg.

A left posterolateral diaphragmatic defect of 5 cm diameter was performed using a 3 mm scissor. Each patch was placed intra-thoracic via a 5 mm port and fixed using non-absorbable silk 4/0 or 5/0 simple interrupted sutures. While placing the dHCM and Gore-Tex® patches, care was taken to place the previously marked side facing the abdominal cavity. Patches were tailored to the size and shape of the diaphragmatic defect. Two dHCM membranes were used in each pig. The dHCM patch was sutured to a plastic o-ring, allowing the membrane to unfold when inside the thorax and adjust to the defect area. Trocar-placement wounds were closed in layers using 2/0 polyglactine suture (Vicryl®; Ethicon, New Jersey, USA), and a running subcuticular 4/0 polyglecaprone suture (Monocryl®; Ethicon). Wounds were covered with a t-shirt to prevent scratching and biting during the post-operative period. Moreover, antibiotic (enrofloxacin, 5 mg/kg, IM) was administrated for 8 days.

Post-operatively, animals were checked for any signs of distress, behavior changes, anorexia or weight loss. When any of these signs was present, human endpoints were applied in order to avoid suffering. After a post-operative period of 7 weeks, animals were euthanized using an overdose of pentobarbital (2000 mg/kg, IV) after administration of ketamine (20 mg/kg, IM) and xylazine (2 mg/kg, IM). In each animal, both hemidiaphragms were collected for further histologic and immunohistochemistry evaluation. Nevertheless, before euthanasia, all animals underwent a thoracoscopic and laparoscopic exploration in order to evaluate adhesion formation and the macroscopic condition of the patches.

For histological and immunohistochemistry evaluation, samples were fixed in 10% formalin, embedded in paraffin and cut to 5 µm sections. H&E and MT staining were performed as previously described in sections II.2.2.1 and II.2.4.1, respectively. Slides were analyzed and scored by an operator blinded to the study groups and outcome at the time of the evaluation. For immunohistochemistry, slides were incubated with rabbit anti-alpha smooth muscle actin 1:50 (#ab5695; Abcam), rabbit anti-CD31 1:50 (#ab28364; Abcam) and mouse anti-CD105 1:100 (#MCA1557F; BioRad) for 2 h at RT. A secondary antibody from the R.T.U. VECTASTAIN® Universal ABC Elite® Kit (#PK-7200, Vector Laboratories) was used in accordance with manufacturer's instructions and revealed with DAB (#SK-

4100, Vector Laboratories). Sections were counterstained with hematoxylin, mounted in an aqueous mounting medium, and observed in an optical microscope with a coupled camera (DM750, Leica).

II-2.5. Microbiology

Microbiology tests are important to determine if a material has antibacterial properties. In chapter VI, dHCM antibacterial properties against *Staphylococcus aureus* (*S. aureus*) were studied, since it is the most prevalent microorganism associated with vascular graft infection [37,38].

For microbiology tests, dHCM were cut in pieces of 2.5 x 2.5 cm² and polypropylene films were used as positive controls. *S. aureus*, (#25923, ATCC) were grown in tryptic soy broth media (TSB; #610053, Frilabo) in a shaking incubator (80 rpm) at 37 °C overnight. Prior to inoculation, bacterial strains were sub-cultured in fresh TBS at 1:50 dilution and incubated for approximately 2 h in a shaking incubator (80 rpm) at 37 °C. A bacterial inoculum of 1 x 10⁴ colony forming units (CFU)/mL was used to completely submerge the membranes in the dish for 1 h at RT. To remove non-attached bacteria, the dish was washed three times with sterile PBS, for 10 s with an 80 rpm rotation and surfaces were dried for 1 h. To identify viable bacteria, contact plates were pressed onto inoculated membranes for 20 s and incubated overnight at 37 °C. After incubation, contact plates were photographed and colonies were counted. The resulting colonies were log transformed and presented as log CFU/ contact plate (2.5 x 2.5 cm²).

II-3. STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism 7 (GraphPad Software, Inc. 2016). The Shapiro–Wilk test was used to assess data normality. When data followed a normal distribution, parametric tests were used, namely the unpaired *t*-test, one-way ANOVA followed by Dunn’s multiple comparison test or two-way ANOVA followed by Tukey’s multiple comparison test. When data did not follow a normal distribution, the Mann–Whitney test was used. When it was necessary to compare survival distributions between groups, Logrank test was used. In chapter VII, GPower version 3.1.9.2 (program written by Franz Faul, Universität Kiel, Germany 1992-2014) was used to calculate the minimum number of animals (effect size 0.7), to obtain a statistical power of 95%. Results were considered statistically significant when $p < 0.05$.

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SECTION 3

DHCM OBTENTION AND CHARACTERIZATION

CHAPTER III

Method to decellularize the human chorion membrane

Chapter III

Method to decellularize the human chorion membrane *

ABSTRACT

The human placenta is considered a biological waste, thus it is a great source of extracellular matrix (ECM) proteins. The human chorion membrane (HCM) is a membrane that composes the human placenta and is constituted by collagens type I, II, IV, V and VI, fibronectin and laminin. To the best of our knowledge, the potential of HCM alone is largely unexplored as a substrate to be used in tissue engineering and regenerative medicine. In this work, we describe, for the first time, the process and method to decellularize the chorion membrane alone. To verify the success of the decellularization protocol, the presence and distribution of cell nuclei and double stranded DNA were quantified and analyzed by DAPI staining, PicoGreen and electrophoresis. After the decellularization protocol an ECM compact and handleably membrane is obtained, the decellularized human chorion membrane (dHCM).

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III-1. INTRODUCTION

Tissue engineering is an interdisciplinary field that aims to develop strategies to replace or regenerate disrupted or injured human tissues or/and organs to restore or establish normal function [1–3]. It consists in a triad of cells, signaling molecules and scaffolds that support and rely upon each other. In fact, scaffolds can be combined with signaling molecules that will provide structural and biochemical cues for guiding cell behavior and tissue regeneration. Scaffolds may be composed by natural and/or synthetic materials and may be obtained through a variety of processing methods [1].

The therapeutic potential of scaffolds derived from the extracellular matrix (ECM) has been widely studied for the regeneration of a range of tissues [4,5] such as musculoskeletal [6–8], cardiovascular [9,10], gastrointestinal [11,12], central nervous system [13], among others [14]. These scaffolds are typically produced through the decellularization of a variety of mammalian tissues [5,13,15,16].

Decellularized matrices can function as substitutes for traditional biomaterials while offering additional advantages of maintaining important properties of the native ECM structure, giving cues for cell modulation and behavior [3,17–19]. These scaffolds are inductive for cell invasion, promoting a constructive remodeling processes that form vascularized, innervated, functional tissue [5,14,18,19]. Moreover, as ECM components are generally conserved among species, decellularized matrix scaffolds are well tolerated even by xenogeneic recipients [15,20,21].

The human placenta is considered a biological waste, but it is also a rich source of ECM proteins [22]. The human chorion membrane (HCM) is located between the amnion (fetal side) and the maternal decidua. [23,24]. It is formed by three different layers (from the fetal to the maternal side): 1) reticular layer that contacts with the amnion and represents the majority of the chorion thickness, composed of collagens I, III, IV, V and VI; 2) basement membrane, which anchors the reticular layer and the trophoblasts and is composed by collagen IV, fibronectin and laminin; 3) trophoblasts layers (3-5 cell layers) that provide nutrients to the embryo by digesting proteins before passing them into the fetal blood [25,26] (Figure III-1).

Although promising, the potential of HCM alone is still largely unexplored as a biological membrane for applications in tissue engineering and regenerative medicine. It has only been used as a reservoir of stem cells [27], pro-angiogenic cells [28] and as a source of small-diameter vascular grafts [22].

Here we describe, for the first time, the decellularization process of the human chorion membrane that results in a compact and handleably membrane.

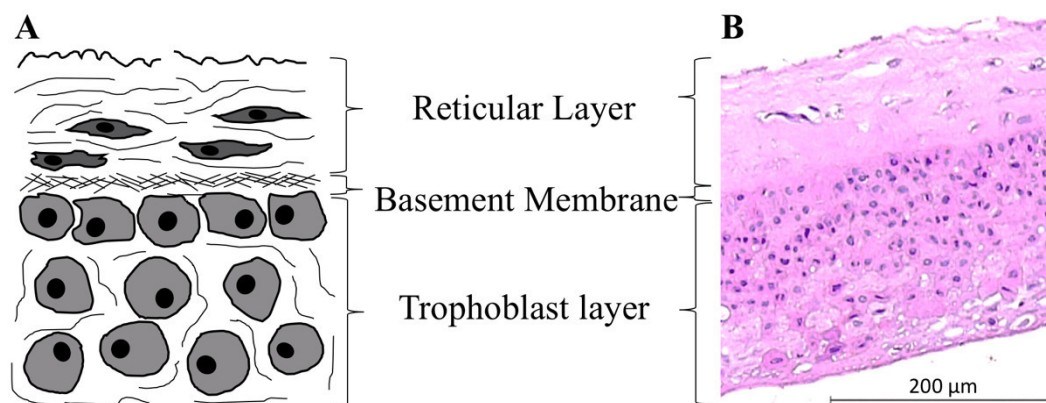


Figure III-1 The human chorion membrane (HCM). (A) Scheme and (B) Hematoxylin/Eosin staining of the native HCM in a transversal cut. The different layers of the HCM – reticular layer, basement membrane, and trophoblast layer - are identified in the figure.

III-2. METHODOLOGY

III-2.1. Overview

Human placentas are collected from elective and term cesarean sections. An informed consent must be signed by all donors. Sterile conditions are maintained during all processing steps and all solutions are sterilized by filtration (0.2 μm - #99500, Techno Plastic Products) or autoclaving. In summary, after collection the placentas are stored in Dulbecco's phosphate-buffered saline (D-PBS - #21600, Gibco) with 10% antibiotic/antimycotic (#15240062, Thermo Fisher Scientific) at 4 °C for a maximum of 1 day. The HCM is removed and separated from the amnion, washed with phosphate buffered saline (PBS - #P4417, Sigma-Aldrich) once to remove the blood and stored at -80°C (Section 4) until the decellularization process (Section 5) for a maximum of 1 year.

Pools of different placentas should be created in order to ensure traceability of the results. The decellularization protocol was optimized by quantifying and analyzing the presence and distribution of cell nuclei and dsDNA by DAPI (#40009, VWR) staining, PicoGreen (#P7589, Invitrogen) and electrophoresis.

III-2.2. Materials

- Human Placenta;
- 350 x 250 x 70 mm Tray (#BOCH8736, VWR);
- Glass container
- Forceps, scissor and scalpel;
- 3 L Buckets (#216-0793, VWR);
- Cell scrapper (#99002, TPP);
- Suture line (#01075503, Minhomedica)
- Filtration system (#99500, TPP)

NOTE: all materials used are previous sterilized

III-2.3. Solutions

- 70% Ethanol;
- D-PBS (#21600, Gibco);
- PBS (#P4417, Sigma-Aldrich);
- 0.5% sodium dodecyl sulfate (SDS) (#MB18101, NZYTech) in ultra-pure water;
- 0.1% SDS in ultra-pure water;
- 1% Triton X-100 (#A16046, Alfa Aesar) in PBS;
- 0.001 mg/mL DNase (#A3778 PanReac AppliChem ITW Reagents) in DNA Reaction Buffer;
- DNA Reaction Buffer: 10 mM Tris-HCl, 2.5 mM MgCl₂, 0.5 mM CaCl₂, pH = 7.6, RT;
- 1-2% and 10% antibiotic/antimycotic (#15240062, Thermo Fisher Scientific) in PBS.

III-2.4. Equipment

- Flow chamber;
- -80 °C freezer;
- 4 °C fridge;
- Water bath at 37 °C;
- Orbital shaker at 110 rpm;
- Weighing machine.

III-2.5. Separate the chorion membrane from human placenta

- 1 - Collect human placentas from elective and term cesarean sections.
 - 2 - Store human placentas in Dulbecco's phosphate-buffered saline (D-PBS) with 10% antibiotic/antimycotic for a maximum of 1 day.
- NOTE: Ideally maintained at 4 °C or store at RT in an ice box.
- 3 - Put human placenta in a sterile tray and separate the less-vascular part of HCM from the placenta with a scissor (Figure III-2A-E).
 - 4 - Remove the amnion membrane from the HCM by gently pushing it (Figure III-2F).
 - 5 - Wash HCM with PBS once to remove the blood excess (Figure III-2G).
 - 6 - Freeze the chorion membrane at -80 °C until further use.

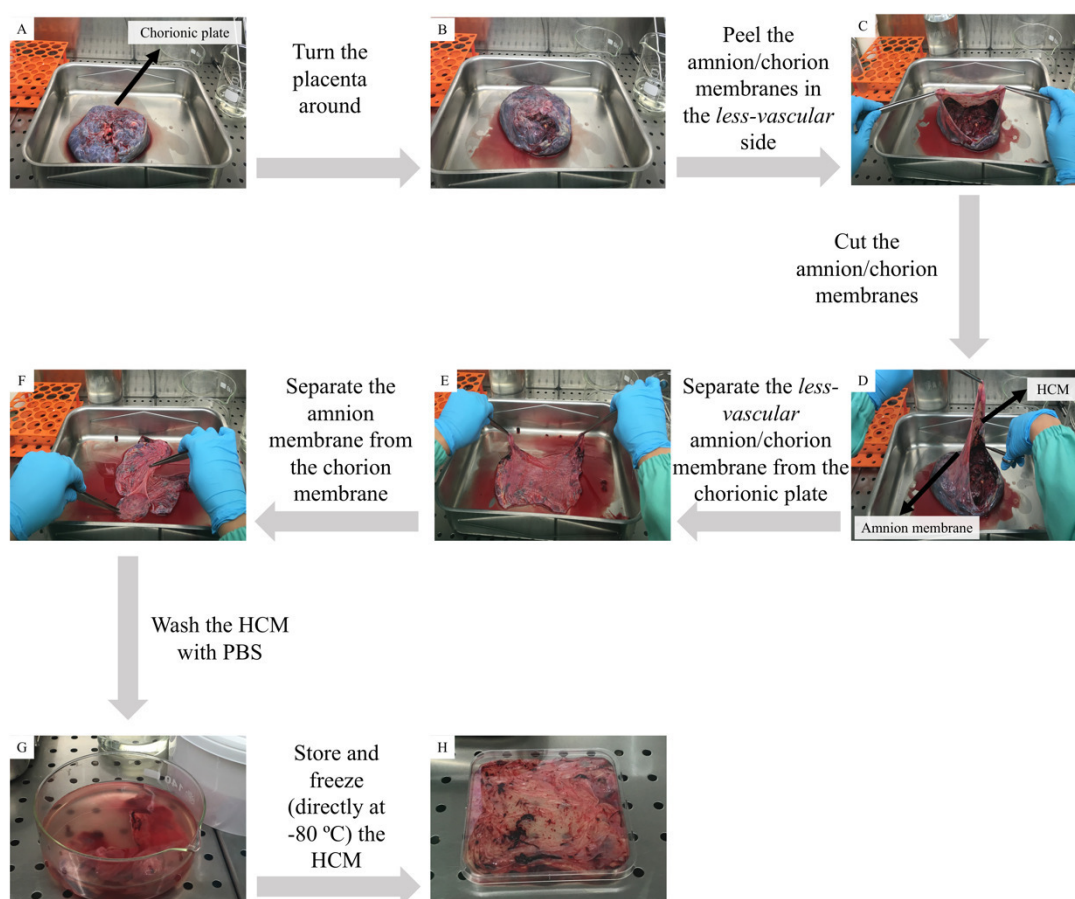


Figure III-2 Separation of the HCM from the human placenta

III-2.6. Decellularization protocol

- 1 - Submit HCM to two freezing/thawing cycles (- 80 °C and 37 °C, respectively - for 20 min each).
 - 2 - Place the membranes in a sterile bucket and perform three washes with 0.5% SDS in ultra-pure water for 2 h at 4 °C in an orbital shaker at 110 rpm. Change the buckets in each wash.
 - 3 - Treat HCM with 0.1% SDS in ultra-pure water overnight, at 4 °C in an orbital shaker at 110 rpm.
 - 4 - Perform three washes with 1% Triton X-100 in PBS for 15 min at 4 °C in an orbital shaker at 110 rpm. Change the buckets in each wash.
 - 5 - Wash the membranes with PBS to remove the detergent for 2 h at 4 °C in an orbital shaker at 110 rpm. Repeat this step at least three times.
 - 6 - Scrape the membranes in both sides (Figure III-3). In order to identify each side of the membrane, knots with a suture line are performed.
- NOTE: In this step the volume of the membranes is considerably lower, due to the removal of the trophoblast layer. Membranes can be switched to a smaller compartment.
- 7 - Treat the membranes with 0.001 mg/mL DNase I in DNA reaction buffer at 37 °C for 30 min.
- NOTE: DNase is maintained in sterile conditions. Only the DNA reaction buffer needs to be filtered.
- 8 - To block the DNase action, wash the membranes with 0.1% SDS in ultra-pure water for 30 min at 4 °C in an orbital shaker at 110 rpm.
 - 9 - Wash the membranes with PBS to remove the detergent for 2 h at 4 °C in an orbital shaker at 110 rpm. Repeat this step at least three times.
 - 10 - Store the membranes in PBS with 1 - 2% antibiotic/antimycotic at 4 °C.

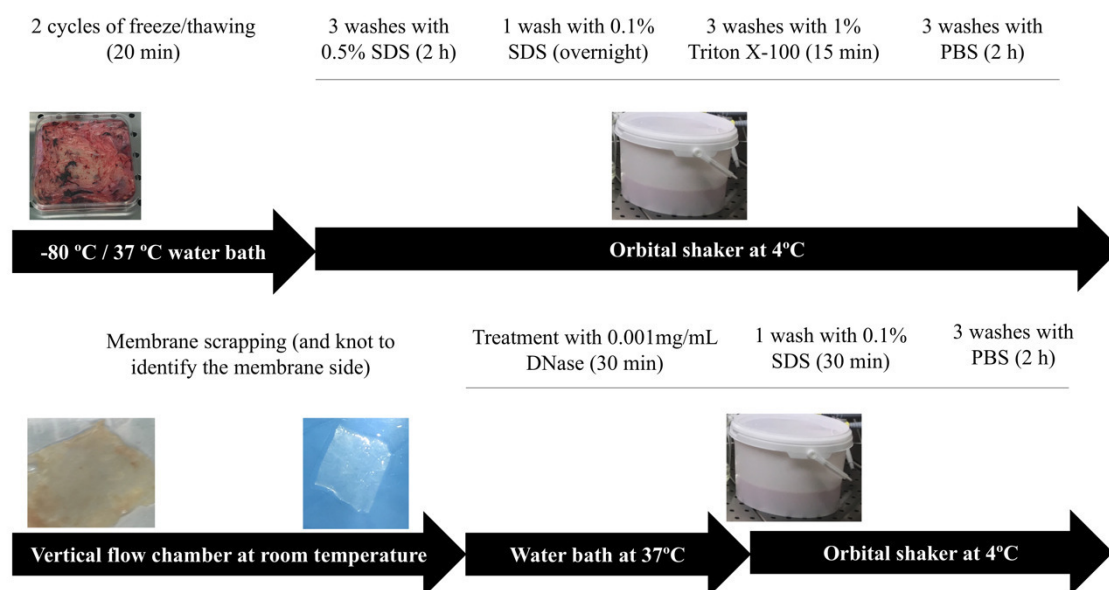


Figure III-3 Scheme of the HCM decellularization protocol.

III-2.7. DNA content measurement

- 1 – Use air-dry decellularized and native membranes.
- 2 – Weight 5 mg of each membrane type.
- 3 – Extract the DNA from both native and decellularized membranes using the DNeasy Blood and Tissue kit (#69504, Qiagen), according to the manufacturer's instructions.
- 4 – Quantify the double-strand DNA (dsDNA) using Quant-IT PicoGreen dsDNA Assay kit (#P7589, Invitrogen) according to the manufacturer's instructions.
- 5 – Perform an electrophoresis, to assess the size of the DNA fragments, using a 1% agarose gel and GeneRuler DNA Ladder Mix (#SM0334, Thermo Fisher Scientific) (Figure III-4).

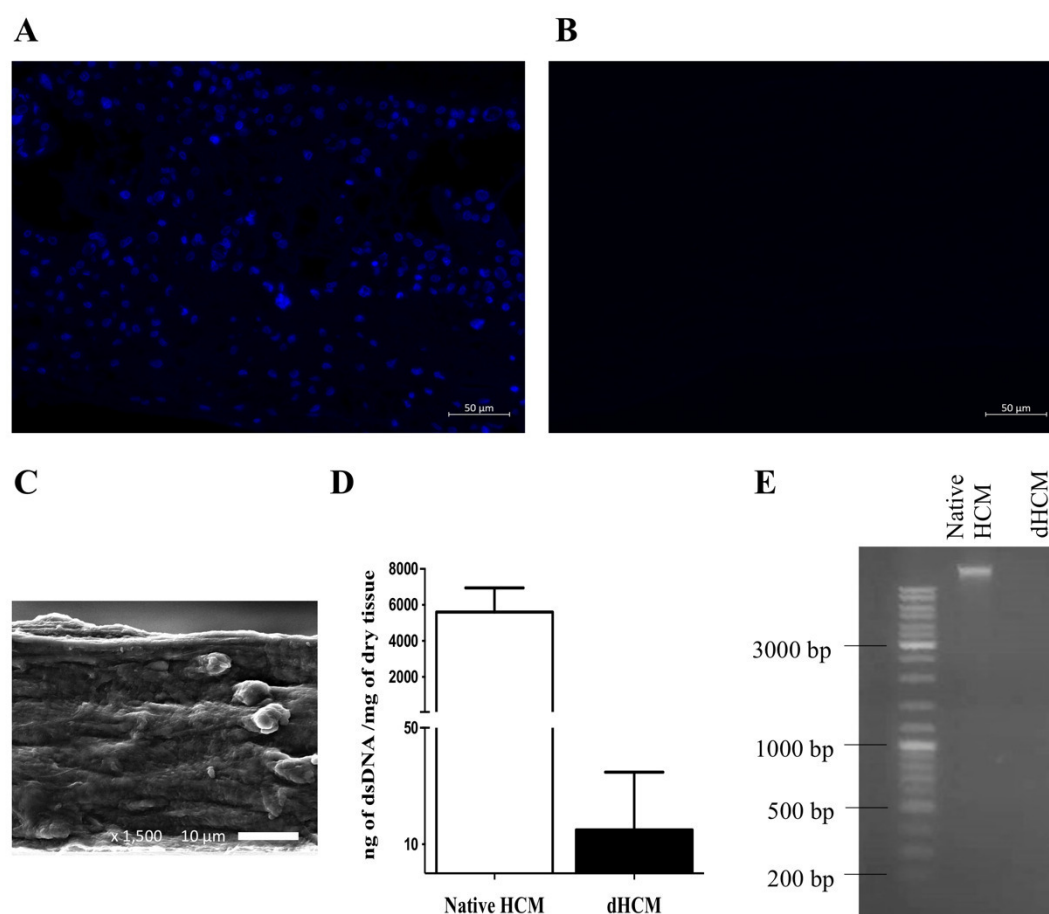


Figure III-4 dHCM characterization. (A-B) Transversal sections of DAPI staining in native HCM (A) and dHCM (B). (C) SEM micrograph of dHCM in a transversal cut. (D) Content of double-stranded DNA in native HCM and dHCM. (E) Electrophoresis in agarose gel of DNA extracted from both native HCM and dHCM.

III-3. PITFALLS AND LIMITATIONS

As all biological tissues, there is some variability between chorion membranes from different donors. Thus, it is very important to use pools of chorion membranes that were obtained from different donors. This variability is observed right from the beginning of the protocol, when the chorion membrane is separated from the human placenta since the less-vascular chorion size may vary between donors (section 4, Figure III-2). Moreover, the consistency of the chorion membranes is not always the same. This is particularly critical during the scrapping step (section 5, Figure III-3). In this step, less consistent membranes, can rupture. This may be a problem if large areas of tissue are needed, forcing the use of bigger placenta pools.

III-4. DISCUSSION

An appropriate decellularization process consists in the removal of cells and cell remnants while retaining the three-dimensional ultrastructure and composition of the ECM [13,14]. In this way it is possible to minimize the immune and inflammatory response to the ECM scaffolds [13]. In fact, there are established criteria for decellularized tissues to avoid host and cell adverse effects that consists in: 1) lack of visible nuclear material (stained with DAPI); 2) DNA fragments with less than < 200 bp; and 3) amount of dsDNA/mg of dry tissue less than 50 ng [29]. Importantly, all these criteria are achieved in the dHCM.

Decellularization methodologies comprise mechanical, chemical, detergent and enzymatic techniques and/or combinations. The method presented in this work comprises a combination of these procedures. These techniques invariably cause a disruption of the ECM architecture, composition and consequently surface ligands [14]. In fact, the dHCM loses the trophoblast layer during the decellularization process.

This protocol starts with two freezing/thawing cycles. This temperature change will form intercellular ice crystals, disrupting the cell membrane and leading to cell lysis [29]. Detergent combinations increase the ECM protein loss [30] and also promote better detergent removal from the decellularized tissue, preventing an adverse immune response *in vivo* [31] and cytotoxicity [32,33]. In this protocol, the ionic detergent SDS is used to solubilize cell and nucleic membranes. Although SDS is associated with the removal of nuclear remnants and cytoplasmatic proteins, it is also related with the removal of glycosaminoglycans and growth factors and with collagen damage. So, it is expected a decrease of this ECM components in the dHCM. In addition to SDS, triton X-100 is also used. Triton X-100 is a non-ionic detergent that disrupts protein-DNA, lipid-lipid, lipid-protein interactions as well as protein-protein interactions, in a milder degree. Like SDS, this detergent removes cellular components and is associated with the disruption and removal of GAGs [29].

The damage and elimination of ECM proteins and DNA from decellularized tissues increases with the exposure time to detergents [34,35] and also with the type of tissue and donor age [29]. In the protocol presented herein, the exposure times to detergents are relatively short but sufficient for cell removal, probably due to the small thickness of the tissue. Moreover, all solutions are used under agitation, facilitating tissue exposure and removal of cellular material. The agitation used in this protocol (110 rpm) is not considered aggressive, avoiding ECM disruption [29].

A physical step consisting in membrane scrapping is also applied in this protocol. In this step the trophoblast layer is removed. By membrane scrapping it is possible to decrease the exposure time to detergents and agitation, preserving the original ECM. After this step, suture knots are important to identify the sides of the membrane without being toxic and harmful for the decellularized membrane. This is a way to control and, eventually, decrease the variability associated with biological tissues.

A treatment with DNase is also performed in order to have a DNA content in accordance with the established criteria for decellularized tissues, as previously described [29]. Nucleases catalyze the hydrolysis of ribonucleotide and deoxyribonucleotide chains, being useful for nucleotides removal after cell lysis [36,37]. However, they are difficult to remove from the tissue and could invoke an immune response. In this protocol a small concentration (0.001 mg/mL) of DNase is used in a reaction buffer to increase its action. In order to successfully remove it from the decellularized tissue, washes with 0.1% SDS (to inhibit its action) and PBS are performed.

Traditionally, the decellularized tissue aims to mimic the original tissue. So, the application of the decellularized tissue is aimed at substituting or to regenerate the original tissue [16,38–42]. It was shown that ECM from different tissues has different compositions, architecture and ligands that will influence cell behavior differently [13]. For example, Crapo and colleagues showed that different cellular responses (including chemotactic and differentiation effects) to central nervous system (CNS) versus non-CNS ECM scaffolds were observed when using PC12 cell line. However, the mitogenic effects were similar in the different scaffolds, suggesting that both scaffolds could support site-appropriate cell phenotype [43].

Oppositely, the purpose of the dHCM is not to mimic the chorion membrane of the human placenta. The dHCM is aimed to mimic biological barriers *in vitro* (such as endothelium and epithelium) by providing a structural support but also chemical cues. This is achieved since an ECM compact and handleably membrane is obtained through the decellularization process. Moreover, we envision the application of this membrane in other fields of tissue engineering and regenerative medicine such as a scaffold to support and/or deliver stem and differentiated cells as well as a surgical patch for tissue regeneration (for example in hernias, wounds, burns and skin defects).

Furthermore, ECM scaffolds are intended to facilitate remodeling of injured tissues *in vivo* [13]. The advantage of using placenta-derived tissues is its privileged immune tolerance [23], allowing a human body implantation without severe immune response.

III-5. CONCLUSIONS

This work describes, for the first time, a decellularization protocol for the HCM alone that results in a compact and handleable membrane. The decellularization protocol was successful since there were lack of visible nuclear material; the DNA fragments had less than 200 bp; and the amount of dsDNA was less than 50 ng/mg of dry tissue. The application of the dHCM is promising to mimic biological barriers *in vitro*, as substrate to support cell growth and/or deliver, and as a surgical patch.

III-6. FUNDING

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III-7. ACKNOWLEDGEMENTS

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Chapter IV

Decellularized Human Chorion Membrane as a Novel Biomaterial for Tissue Regeneration

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ABSTRACT

Although some placenta-derived products are already used for tissue regeneration, the human chorion membrane (HCM) alone has been poorly explored. In fact, just one study uses a decellularized HCM (dHCM) with native tissue architecture (i.e. without ECM suspension creation) as a substrate for cell differentiation. The aim of this work is to fully characterize the dHCM for the presence and distribution of cell nuclei, DNA, and ECM components. Moreover, the mechanical properties, the *in vitro* biological performance and *in vivo* biocompatibility were also studied. Our results demonstrated that the HCM was successfully decellularized and the main ECM proteins were preserved. The dHCM has two different surfaces, the reticular layer side and the trophoblast layer side; and is biocompatible both *in vitro* and *in vivo*. Importantly, the *in vivo* experiments demonstrated that on day 28 the dHCM starts to be integrated by the host tissue. Altogether these results support the hypothesis that dHCM may be used as a biomaterial for different tissue regeneration strategies, particularly when a membrane is needed to separate tissues, organs or other biologic compartments.

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IV-1. INTRODUCTION

The human placenta is usually considered a biological waste. Nevertheless, it is consistently available from full-term births and it is also a source of mesenchymal stem cells, growth factors and extracellular matrix (ECM) proteins [1,2].

Some placenta-derived products are already used for tissue regeneration. Nevertheless, when non-decellularized they are only used in poorly vascularized sites, such as some wounds, tendon, ligament and cartilage [3]. So, one way to obtain ECM proteins and avoid a host adverse immune response when implanted *in vivo*, is through tissue decellularization [4]. In fact, the human placenta has been fully decellularized through the creation of ECM suspensions to further create membranes [5] or sponges [6]. In these works, the various components of the human placenta are not differentiated, such as the amnion (HAM) and the chorion membrane (HCM) [7]. Moreover, the native tissue architecture is not maintained. Nevertheless, some works maintained the native tissue architecture after decellularization, for the whole placenta [8] and for the HAM [9–17] and HCM alone [18].

Decellularized HAM alone is widely characterized and studied and has been shown to induce osteogenic differentiation of human dental apical papilla cells [9], to be a good chondrocyte substrate/carrier [10], and to promote epithelization [11–13]. Moreover, decellularized HAM was used in cell-matrix adhesion studies, to produce skin equivalents, and as a pericardial substitute [12,14–17]. On the other hand, HCM alone as a biomaterial for tissue regeneration is still largely unexplored. Although, HCM has been used as a reservoir of stem [19] and pro-angiogenic [20] cells, and as a source of small-diameter vascular grafts [1], only one study uses a decellularized HCM (dHCM) with native tissue architecture (i.e. without ECM suspension creation) as a substrate for cell differentiation [18]. However, in that study, the dHCM is poorly characterized for the presence of ECM components, mechanical properties, *in vitro* cytocompatibility and *in vivo* biocompatibility.

The purpose of this study is to fully characterize the dHCM, for the presence and distribution of cell nuclei, DNA, and ECM components. Finally, the mechanical properties, the *in vitro* and *in vivo* biocompatibility of dHCM were characterized envisioning its use as an ECM-based scaffold able to guide tissue regeneration, particularly when a membrane is needed to separate tissues, organs or other biologic compartments.

IV-2. MATERIALS AND METHODS

IV-2.1. Human Placentas

Human placentas used in this study were collected from elective and term cesarean sections performed on the Obstetrics Department of the Hospital de Braga. The study follows a protocol approved by the ethical committee of the Hospital de Braga and of the University of Minho (CESH 030/2016, SECVS 136/2015, respectively) and following all the international best practices for research with human biological samples. An informed consent was signed by all the donors. Sterile conditions were maintained during all processing steps. Placentas were stored in Dulbecco's phosphate-buffered saline (D-PBS) with 10% antibiotic/antimycotic (#15240062, Thermo Fisher Scientific) at 4 °C for a maximum of 1 day. HCM was separated from the amnion, washed with phosphate buffered saline (PBS) to remove any blood and stored at - 80°C until further use.

IV-2.2. Decellularization of human chorion membrane (HCM)

As previously described by us [21], HCM were submitted to two freezing/thawing cycles (- 80 °C and 37 °C, respectively) and, subsequently, were treated with different concentrations (0.5% and 0.1%) of sodium dodecyl sulfate (SDS) (#MB18101, NZYTech) solution in ultra-pure water. HCM were submitted to three washes with 0.5% SDS during 2 h each. Then, the membranes were washed overnight with 0.1% SDS. Three washes of 15 min were performed with 1% triton-X100 (#A16046, Thermo Fisher Scientific) in PBS. All treatments were done at 4 °C in an orbital shaker at 110 rpm. The membranes were then scrapped in both sides and the trophoblast layer (TL) was removed and further used for collagen and glycosaminoglycans (GAGs) quantification. In order to identify both membrane sides (trophoblast and reticular layer sides), the membranes were marked with a knot with suture line. Subsequently, membranes were treated with 0.001 mg/mL DNase I (#A3778 PanReac AppliChem ITW Reagents), for 30 min at 37 °C, followed by a 30 min wash with 0.1 % SDS at 4 °C. Finally, the membranes were washed with PBS at least three times, during 2 h each, at 4 °C. All the process was done under sterile conditions. After decellularization, the membranes were stored in PBS with 1-2% antibiotic/antimycotic at 4 °C.

IV-2.3. DNA extraction and quantification

Membranes were air dried and weighed and total DNA from both native and decellularized HCM (dHCM) was extracted using the DNeasy Blood and Tissue kit (#69504, Qiagen), according to manufacturer's instructions. The quantification of double-strand DNA (dsDNA) was performed using Quant-IT PicoGreen dsDNA Assay kit (#P7589, Invitrogen) according to manufacturer's instructions. Electrophoresis was performed to assess the size of the DNA fragments, using a 1% agarose gel and GeneRuler DNA Ladder Mix (#SM0334, Thermo Fisher Scientific). Four independent samples were used in each condition.

IV-2.4. Histological analysis

The samples were fixed in 10% neutral-buffered formalin at 4 °C (for at least 24 h), embedded in paraffin and 5 µm thick transverse sections were cut with a microtome. Histochemical stains such as hematoxylin and eosin (H&E) and Masson's trichrome were performed in the samples. For H&E staining, samples were stained with hematoxylin (#7212, Thermo Fisher Scientific) for 1 min, washed for 30 s and stained with eosin for 10 min (#71204, Thermo Fisher Scientific). For Masson's trichrome staining, slides were submitted to Azure B solution for 5 min, stained with hematoxylin for 5 min and washed in picric ethanol for 5 min. After that, samples were stained in Biebrich Scarlet-Acid Fuchsin for 15 min, submitted to 1% phosphomolybdic acid for 5 min, and to Aniline Blue for 4 min. After each staining, all slides were washed with water, let to dry, and rinsed with alcohol, cleared in xylene, and mounted in entellan rapid (#107960, Merck). Slides were observed under an optical microscope with a coupled digital camera (DM750, Leica). At least, three independent samples were used in each condition.

IV-2.5. Scanning electron microscopy (SEM)

Samples were fixed with 2.5% glutaraldehyde in PBS. After three PBS washes, samples were dehydrated with increasing concentrations of ethanol (10% to 100%). Samples were air dried and mounted in SEM pins using carbon tape. The samples were coated with gold using a Sputter Coater (#EM ACE600, Leica). Micrographs were collected with a Scanning Electron Microscope with EDS (#JSM-6010 LV, JEOL). At least, three independent samples were used in each condition.

IV-2.6. Swelling assay

Initially, dry samples were weighted and then were immersed in D-PBS or culture medium. At different time-points (0h30 min; 1h30 min; 2h30 min; 3h30 min; 4h30 min; 5h30 min), samples were weighted immediately after the excess of liquid was removed by putting them between two pieces of filter paper. Samples were re-immersed in liquid until the next time-point. The process was repeated until the equilibrium was reached (stable percentage of water uptake). Six samples were used in each condition.

IV-2.7. Collagen quantification

The collagen content of HCM, dHCM and trophoblast layer (TL) was extracted and quantified using Sircol Collagen Assay Kit (#S5000, biocolor) for soluble collagen and Sircol Insoluble Collagen Assay kit (#2000, biocolor) as described before [22] and according to manufacturer's instructions. Three independent samples were used in each condition.

IV-2.8. Sulfated glycosaminoglycans quantification

Sulfated glycosaminoglycans (GAGs) content of HCM, dHCM and TL was quantified as previously described [23]. Samples were digested with 0.5 mg/mL papain (#P4762, Sigma-Aldrich) and the supernatant was stained with 1,9-dimethylmethylene blue (DMB). A dilution series of chondroitin sulfate in distilled water (50 µg/mL) was used as standard solution. Samples were diluted 1:10 in distilled water before measurement. Twenty µL of standards and diluted samples were mixed with 250 µL of DMB in a 96-well plate and absorbance was measured immediately at 525 nm using a microplate reader (SynergyHT, Biotek). Three independent samples per condition were analyzed.

IV-2.9. Protein extraction and quantification

Soluble protein was extracted from HCM and dHCM using Tissue Extraction Reagent I (#FNN0071, Thermo Fisher Scientific) with a protease inhibitor cocktail (#P8340, Sigma-Aldrich). The soluble protein content was determined using Protein Assay Dye Reagent Concentrate (#5000006, Biorad) according to manufacturer's instructions.

IV-2.10. SDS-PAGE

The SDS Gel Preparation Kit (#08091, Sigma-Aldrich) was used to prepare the 4% stacking gel and 9% running gel. For each sample, 0.5 µg/µL of protein were loaded in the respective well. Following SDS-PAGE, the gel was stained with Coomassie blue R-250 (HS-604, National Diagnostics) and image was obtained using a Transilluminator (Biospectrum ac chemi hr 410, UVP). At least, three independent samples were used in each condition.

IV-2.11. Dot-Blot

One drop of each sample of soluble protein was placed in a nitrocellulose membrane. After drying, membranes were incubated for 1 h with 5% BSA, with agitation, at RT. Subsequently, membranes were incubated with primary antibody: mouse anti-collagen type I 1:1000 (#ab90395, abcam); rabbit anti-collagen type IV, 1:500 (#ab6311, abcam); rabbit anti-fibronectin 1:500 (#ab45688, abcam); and mouse anti-laminin, 1:500 (#L8271, Sigma-Aldrich). After overnight incubation, membranes were washed 3x for 5 min with TBS-tween 20, and then R.T.U. VECTASTAIN® Universal ABC Elite® Kit (#PK-7200, Vector Laboratories) was used as a secondary antibody, in accordance with manufacturer's instructions. Finally, incubation was revealed using Peroxidase Substrate Kit (DAB) (#SK-4100, Vector Laboratories). Extraction buffer without samples was used as a negative control. Collagen type I (#sc-136157, Santa Cruz Biotechnology), collagen type IV (#C5533, Sigma-Aldrich), fibronectin (#FC010, Sigma-Aldrich), and laminin (#L6274, Sigma-Aldrich) were used as positive controls. At least, three independent samples were used in each condition.

IV-2.12. Immunolocalization of proteins

Immunolocalization of different proteins, such as collagen type I, collagen type IV, fibronectin and laminin, was performed in paraffin-embedded samples sectioned at 5 µm, as previously described for collagen type II [24]. Briefly, samples were incubated with mouse anti-collagen type I 1:100 (#ab90395, abcam); rabbit anti-collagen type IV, 1:50 (#ab6311, abcam); rabbit anti-fibronectin 1:300 (#ab45688, abcam); and mouse anti-laminin, 1:300 (#L8271, Sigma-Aldrich), overnight at 4 °C in a humidified atmosphere. As a secondary antibody, R.T.U. VECTASTAIN® Universal ABC Elite® Kit (#PK-7200, Vector Laboratories) was used, in accordance with manufacturer's instructions. Incubation was revealed using Peroxidase

Substrate Kit (DAB) (#SK-4100, Vector Laboratories). Samples were counterstained with hematoxylin and mounted in an aqueous mounting medium. Slides were observed in an optical microscope with a coupled camera (DM750, Leica). At least, three independent samples were used in each condition.

IV-2.13. Mechanical properties

Static mechanical properties were assessed using Universal mechanical testing equipment (#5543, INSTRON) equipped with a 1 kN load cell. Nine samples of HCM and dHCM were cut in pieces of 20 mm x 5 mm and mounted in specific cassettes (to prevent the clamping system from damaging the samples) and the specimens were hydrated. The strain rate was defined at 5 mm/min and a 10 mm gauge length was used in tensile tests. Tests were finished when the specimens were ruptured.

IV-2.14. Cytotoxicity analysis

Human umbilical vein endothelial cell line EA.hy926 and human lung fibroblast cell line MRC-5 were cultured in complete medium (DMEM with 10% fetal bovine serum (FBS; #A3160801, Thermo Fisher Scientific) and 1% Penicillin/Streptomycin (#15240062, Thermo Fisher Scientific)) in T150 flasks at 37 °C in a humidifier incubator with 5% CO₂. After reaching 80% of confluence, the cells were seeded into inserts. dHCM were mounted in cell crown inserts for 24-well plates (#Z742380-12EA, Sigma). Millicell hanging cell culture inserts, PET 0.4 µm for 24-well plates (#MCHT24H48, Millicell) were used as control. Both inserts were immersed in culture medium overnight before cell seeding. EA.hy926 and MRC-5 cells were seeded on the inserts with a density of 20x10³ cells/cm². Metabolic activity was evaluated by MTS assay (CellTiter 96 AQueous One Solution, Promega). Three independent assays for each cell line were performed.

IV-2.15. *In vivo* studies

IV-2.15.1. Animals and ethical issues

The experiments were performed in male Balb/c mice with 9 weeks old (Charles Rivers, Barcelona, Spain) and a total of 20 animals were used. Animals were housed five per cage in a limited-access rodent facility. Food and water were available *ad libitum* and animals were maintained in a climate-controlled room (22.0

± 0.5 °C of temperature) in 12/12 h light/dark cycle with lights on at 8:00 am. The experimental protocol was approved by the Institutional Ethical Commission and followed the European Community Council Directive concerning the use of animals for scientific purposes. Before surgery, animals were exposed to handling twice a day. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

IV-2.15.2. Subcutaneous implantation

The main goal of this *in vivo* study was the assessment of the biocompatibility of dHCM membranes in healthy animals. Balb/c mice were anesthetized by an intraperitoneal injection of Domitor (Medetomidine 0.5 mg/Kg) plus Imalgene (Ketamine 75 mg/kg). The dorsum of the animals (implantation area) was shaved, cleaned and sterilized with betadine®, and two skin incisions were made (each 0.5 cm length) in each mouse. A sample of the dHCM was implanted subcutaneously in each of the pockets and the incision was closed using 5-0 sutures (15 animals, 5 animals for each time-point). Empty defects were used as controls, either in the control group (5 animals, 2 animals for the first two time-points and 1 animal in the last time-point) and in the contralateral side of the pocket containing the material. After the procedure, the anesthesia was reversed with a subcutaneous injection of anti-sedan and the animals were monitored until fully awake (eating and grooming). After 4, 10, or 28 days, animals were euthanized by pentobarbital injection, and dHCM membranes and surrounding tissues were explanted for subsequent histological analyses.

IV-2.16. Statistical analysis

Statistical analysis was performed using Graph Pad Prism 7. Shapiro-Wilk test was used to assess data normality. When data followed a normal distribution, parametric tests were used, namely unpaired t-test and two-way ANOVA test followed by Turkey's multiple comparisons test. When data did not follow a normal distribution, the Mann-Whitney test was used. A $p < 0.05$ was considered statistically significant.

IV-3. RESULTS

IV-3.1. HCM decellularization and ECM protein retention

The efficiency of the decellularization protocol was assessed and the results are summarized in Figure IV-1. In the H&E stainings no nuclei can be observed in the dHCM when compared with native tissue (Figure IV-1A, IV-1B). Moreover, the chemical and physical processes used in the decellularization process completely removed the HCM's trophoblast layer and the nuclei from the reticular layer (Figure IV-1A, IV-1B). SEM images revealed that the dHCM is composed by nanofibers in the reticular layer side (Figure IV-1C), however, a thin compact layer covered the dHCM in the trophoblast layer side of the membrane (Figure IV-1D). Cellular removal in the dHCM (Figure IV-1F) was also corroborated by DAPI staining in which no signal is detected compared with the native membrane (Figure IV-1E). Additionally, DNA quantification and length of DNA fragments showed a significant removal of DNA ($p = 0.0002$) in dHCM when compared with HCM (Figure IV-1G). The dHCM presented a DNA content around 10 ng of dsDNA/mg of dry tissue (Figure IV-1G). DNA content of dHCM was so low that no DNA was visible on the agarose gel in lanes loaded with dHCM, compared with the large amounts of DNA in lanes loaded with native tissue (Figure IV-1H). Thickness measurement of air-dried HCM and dHCM was also performed. After the decellularization protocol the membrane became 5 times thinner ($119.50 \pm 34.32 \mu\text{m}$ of HCM compared to $24.50 \pm 3.11 \mu\text{m}$ of dHCM) ($p = 0.0150$; Figure IV-1I). Although, having large differences in tissue thickness between native and decellularized tissue, a compact and easy to manipulate membrane was obtained (Figure IV-1J, IV-1K).

The swelling behavior of dHCM in culture medium and PBS is shown in Figure IV-1L. In the first 30 min, an increase of 230 – 240% of dHCM's weight was observed. After this time, the swelling behavior stabilized around 300 – 350%. Moreover, when wet, dHCM's thickness increased to $36.00 \pm 8.03 \mu\text{m}$ ($p = 0.0159$; data not shown).

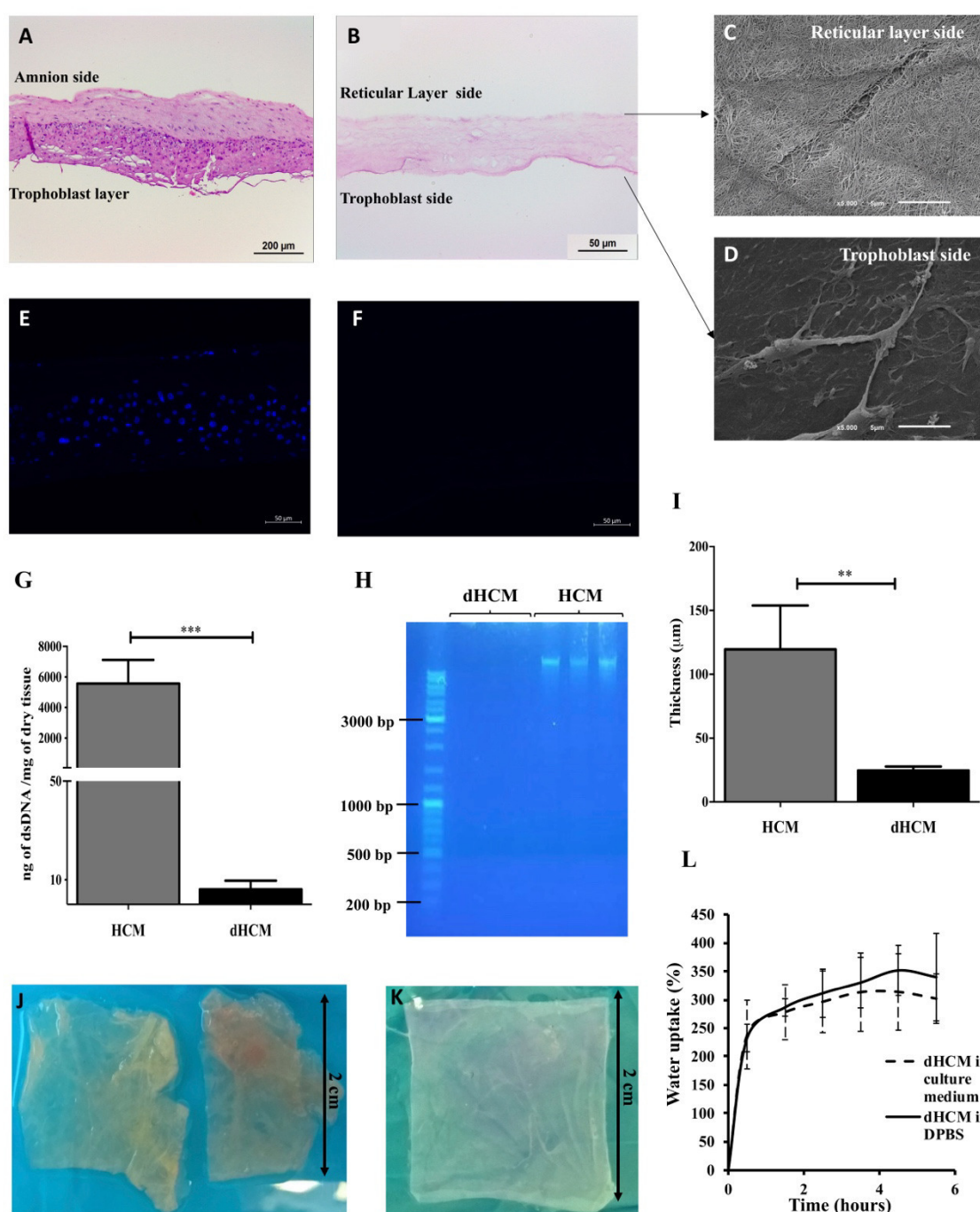


Figure IV-1 Human chorion membrane decellularization. Representative transversal sections of Hematoxylin/Eosin (H&E) staining of HCM (**A**) and dHCM (**B**). Representative SEM micrographs of dHCM from the reticular layer side (**C**) and the trophoblast layer side (**D**). Representative transversal sections of DAPI staining from HCM (**E**) and dHCM (**F**). Double-stranded DNA (dsDNA) quantification in HCM and dHCM (**G**). Agarose gel electrophoresis of DNA extracted from HCM and dHCM (**H**). Thickness of air-dried HCM and dHCM measured with a picometer in, at least, three different sites (**I**). Top view of HCM (**J**) and dHCM (**K**) cut into pieces of 2 cm x 2 cm. Swelling behavior of dHCM in culture medium and D-PBS (**L**). Where, ** $p \leq 0.01$ and *** $p \leq 0.001$.

The dHCM's composition was assessed using SDS-PAGE, dot blot and immunolocalization of proteins. SDS-PAGE gel showed similarity among dHCM samples (Figure IV-2A), suggesting that the decellularization protocol is reproducible. Moreover, when compared to HCM, dHCM seems to preserve the higher molecular weight species (Figure IV-2A). By dot blot, it was possible to confirm the presence of some ECM high molecular weight proteins, such as collagen type I, collagen type IV, fibronectin, and laminin, in both native and decellularized tissues (Figure IV-2B). To assess the distribution of these proteins, tissue sections of HCM and dHCM were used. As observed in Figure IV-2C, while collagen type I was spread along the dHCM, collagen type IV, fibronectin, and laminin were focused on dHCM's trophoblast layer side. Quantitative analysis was performed to detect the presence of soluble and insoluble collagen and sulfated glycosaminoglycans (sGAG) content in HCM, dHCM, and trophoblast layer (TL). Regarding soluble collagen (Figure IV-2D), significant differences were observed between the HCM, dHCM and TL ($p = 0.0045$ (HCM *vs.* dHCM); $p < 0.0001$ (HCM *vs.* TL); $p = 0.0006$ (dHCM *vs.* TL)). Soluble collagen content was 6.33 ± 0.92 $\mu\text{g}/\text{mg}$ of dry tissue in HCM, 3.70 ± 0.46 $\mu\text{g}/\text{mg}$ of dry tissue in dHCM and, 0.80 ± 0.06 $\mu\text{g}/\text{mg}$ of dry tissue in TL. This tendency was not observed in insoluble collagen content (Figure IV-2E), where dHCM was associated with a higher quantity of insoluble collagen (137.90 ± 19.68 $\mu\text{g}/\text{mg}$ of dry tissue) when compared with HCM (68.51 ± 18.51 $\mu\text{g}/\text{mg}$ of dry tissue, $p < 0.0001$) and TL (55.09 ± 20.42 $\mu\text{g}/\text{mg}$ of dry tissue, $p < 0.0001$). No statistically significant differences were observed between HCM and TL ($p = 0.2850$). The total collagen (soluble and insoluble) amount was significantly higher in dHCM (142.30 ± 20.07 $\mu\text{g}/\text{mg}$ of dry tissue) when compared with HCM (74.78 ± 17.60 $\mu\text{g}/\text{mg}$ of dry tissue, $p < 0.0001$) and TL (54.66 ± 20.92 $\mu\text{g}/\text{mg}$ of dry tissue, $p < 0.0001$). No differences were observed between HCM and TL ($p = 0.1406$). For sulfated GAG quantification (Figure IV-2F), dHCM presented the lowest content (2.64 ± 0.19 $\mu\text{g}/\text{mg}$ of dry tissue) when compared with HCM (17.07 ± 1.08 $\mu\text{g}/\text{mg}$ of dry tissue, $p < 0.0001$) and TL (6.45 ± 2.09 $\mu\text{g}/\text{mg}$ of dry tissue, $p = 0.0040$). Sulfated GAGs content in HCM was also significantly higher than in TL ($p < 0.0001$).

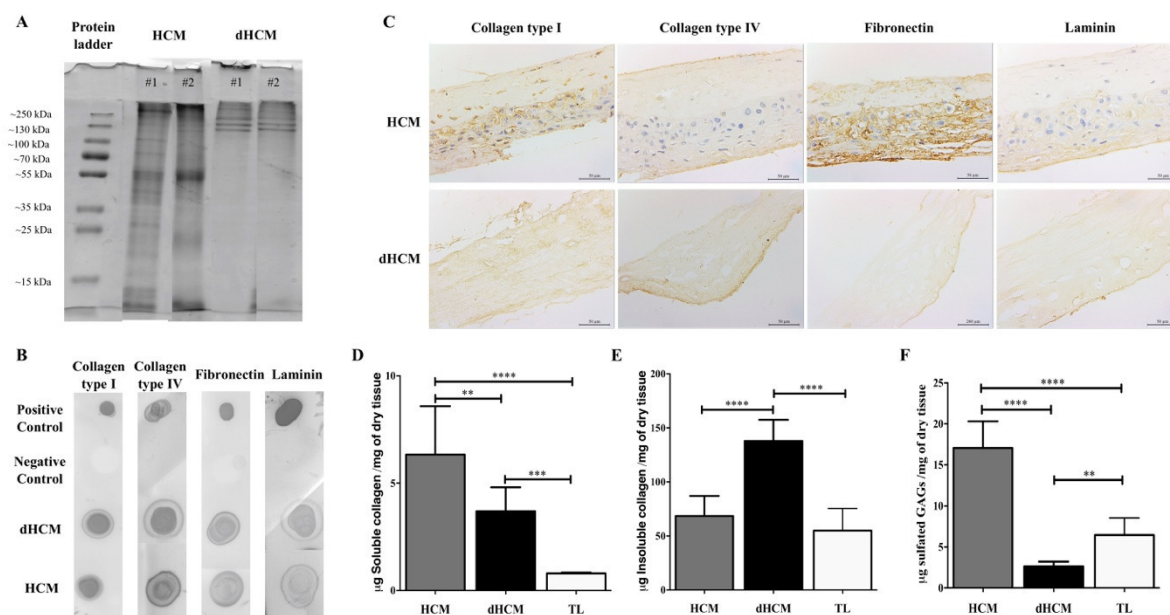


Figure IV-2 dHCM protein composition. Representative lanes of two different samples from an SDS-PAGE gel of the digested HCM and dHCM (**A**). Representative dot blot results for collagen type I, collagen type IV, fibronectin and laminin, in both native and dHCM tissues. Digestion buffer without samples was used as a negative control, while the native protein was used as a positive control (**B**). Immunolocalization of collagen type I, collagen type IV, fibronectin and laminin in representative transversal sections of HCM and dHCM (**C**). Quantification of soluble collagen (**D**), insoluble collagen (**E**), and sulfated glycosaminoglycans (GAGs) (**F**) content in HCM, dHCM, and TL in µg/mg of dry tissue. Where ** $p \leq 0.01$; *** $p \leq 0.001$ and **** $p \leq 0.0001$.

IV-3.2. dHCM's mechanical properties

Mechanical properties were assessed in hydrated samples. Nine stress-strain curves are represented in Figure IV-3A and IV-3B for both native and decellularized tissue, respectively. At low strain levels, the stress varied linearly with the strain in accordance with Hooke's law. At higher strain levels, membranes show a strain hardening behavior. The average ultimate tensile strength was 1.80 ± 0.28 MPa for native tissue and 5.33 ± 0.41 MPa for dHCM (Figure IV-3C). The average Young's Modulus was 1.69 ± 0.19 MPa for the native and 5.94 ± 0.47 MPa for the decellularized tissue (Figure IV-3D). Both, ultimate tensile strength and Young's Modulus were significantly higher in dHCM ($p < 0.0001$).

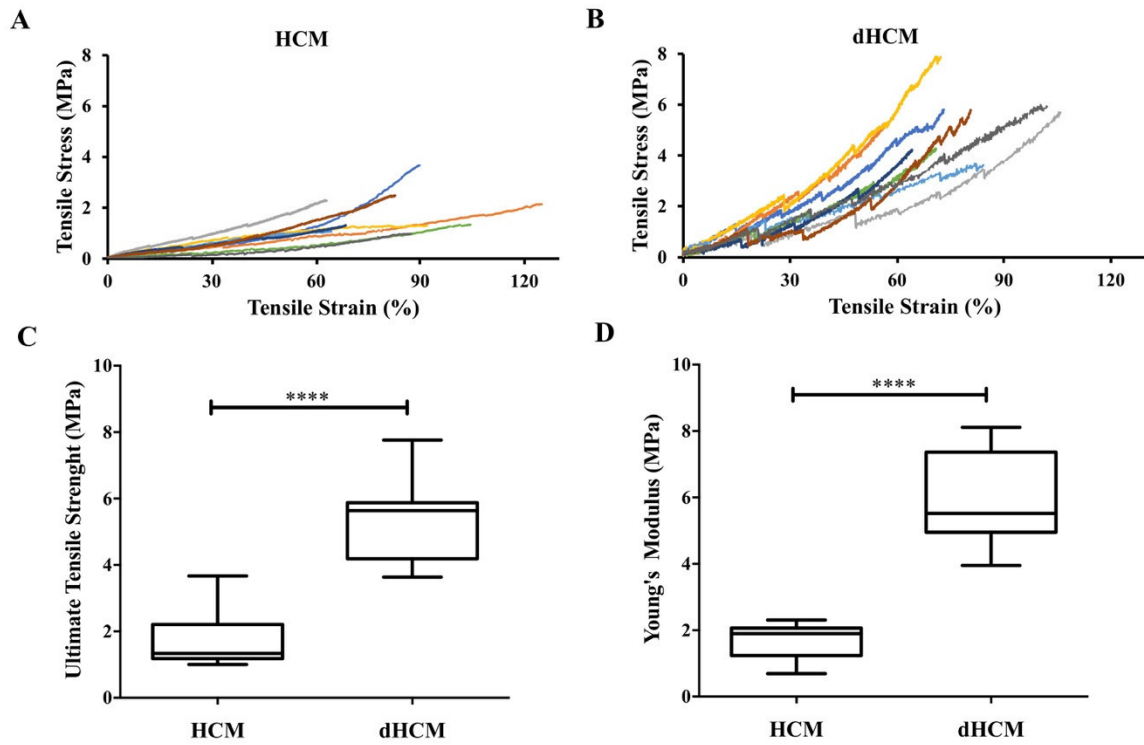


Figure IV-3 Mechanical properties of native and decellularized HCM. Stress-strain curves of HCM (A) and dHCM (B). Ultimate tensile strength (C) and Young's modulus (D) of both HCM and dHCM. Where **** $p \leq 0.0001$.

IV-3.3. Biocompatibility of dHCM *in vitro* and *in vivo*

To evaluate the biocompatibility of dHCM *in vitro*, metabolic activity of EA.hy926 and MRC-5 cell lines were analyzed. Commercial inserts were used as controls (CTR). In all conditions, the metabolic activity of both EA.hy926 (Figure IV-4A) and MRC-5 (Figure IV-4B) cell lines increased over time. In general, both cell lines behaved in the same way. Regarding day 1, no differences were observed between conditions with EA.hy926 cell line ($p = 0.8480$ (CTR *vs.* dHCM trophoblast layer side); $p = 0.9636$ (CTR *vs.* dHCM reticular layer side); $p = 0.7012$ (dHCM trophoblast layer side *vs.* dHCM reticular layer side)) and with MRC-5 cell line ($p = 0.9069$ (CTR *vs.* dHCM trophoblast layer side); $p = 0.3545$ (CTR *vs.* dHCM reticular layer side); $p = 0.1397$ (dHCM trophoblast layer side *vs.* dHCM reticular layer side)). On day 3, dHCM reticular layer side presented a significantly lower metabolic activity in both EA.hy926 ($p = 0.0277$ (*vs.* dHCM trophoblast layer side)) and MRC-5 cell line ($p < 0.0001$ (*vs.* dHCM reticular layer side); $p =$

0.0061 (*vs.* CTR)). Nevertheless, no differences were observed between CTR and both dHCM sides in EA.hy926 cell line ($p = 0.1997$ (*vs.* dHCM trophoblast layer side); $p = 0.5962$ (*vs.* dHCM reticular layer side)) and between CTR and dHCM trophoblast layer side in MRC-5 cell line ($p = 0.1365$). Regarding day 6, CTR condition outperformed both dHCM sides with EA.hy926 ($p < 0.0001$ (*vs.* dHCM trophoblast layer side); $p < 0.0001$ (*vs.* dHCM reticular layer side)) and MRC-5 cell line ($p < 0.0001$ (*vs.* dHCM trophoblast layer side); $p < 0.0001$ (*vs.* dHCM reticular layer side)). No differences were observed between dHCM sides ($p = 0.8383$ (EA.hy926); $p = 0.9756$ (MRC-5)). To further evaluate the biocompatibility of dHCM *in vivo*, dHCM membranes were subcutaneously implanted in balb/c mice. After 4, 10 and 28 days, animals were sacrificed and the tissue surrounding the dHCM membranes was collected for histological characterization. All animals survived during the period of the *in vivo* experiments. These animals did not demonstrate any mortality and infection throughout the study period, and the implantation of dHCM membranes did not produce any surgery-related and implantation-related abnormalities. As observed on Figure IV-4C, H&E staining demonstrated that on day 4, dHCM was surrounded by a thin layer of cells, however no inflammatory cells were identified. Masson's trichrome staining showed that the cell layer surrounding dHCM was also composed by collagen fibers (Figure IV-4C). In fact, this fibrotic capsule, composed by fibroblasts and collagen fibers, increased on day 10. Regarding day 28, an integration of native cells into dHCM was observed.

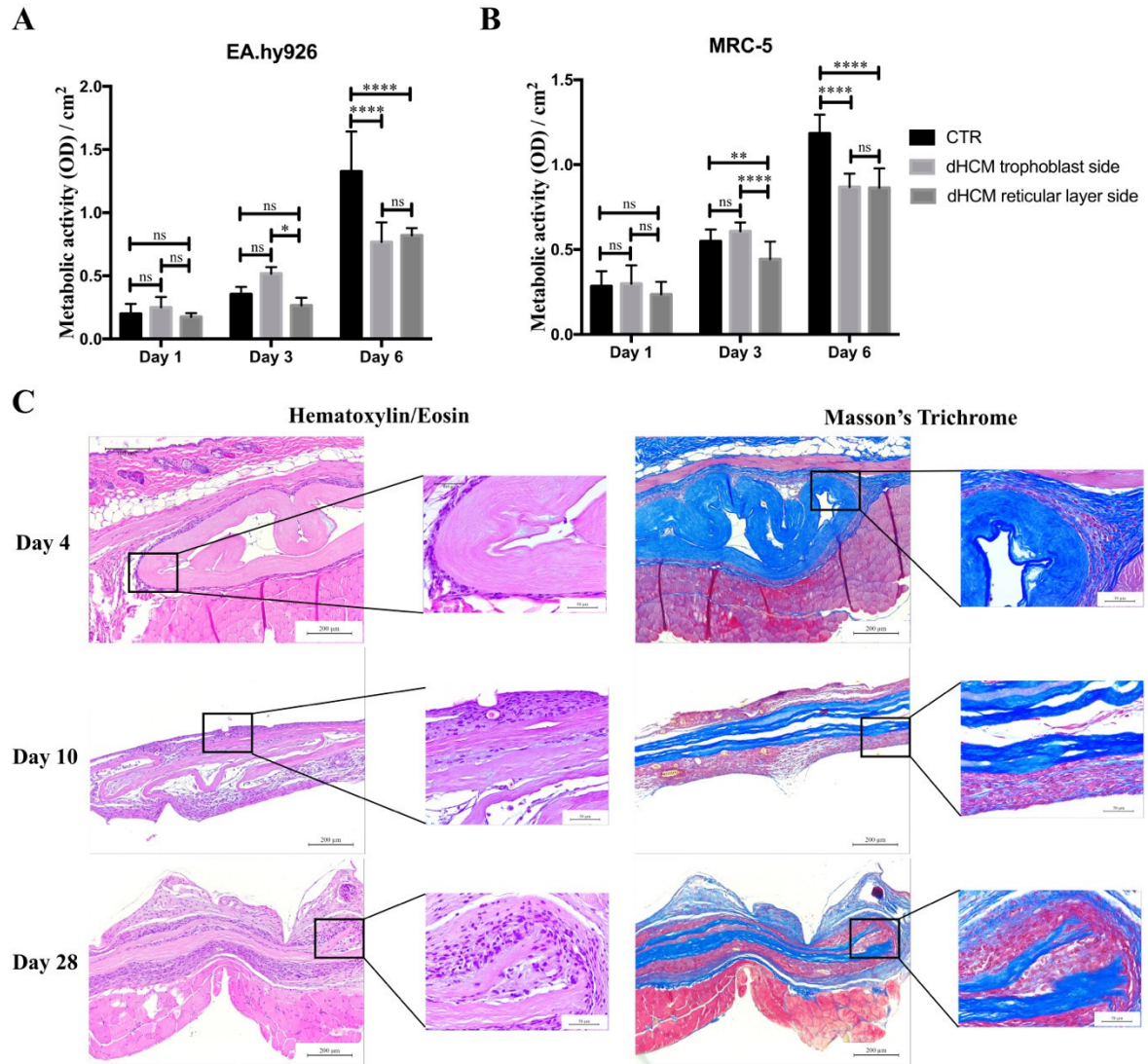


Figure IV-4 Mechanical properties of native and decellularized HCM. EA.hy926 **(A)** and MRC-5 **(B)** cells' metabolic activity in commercial inserts (CTR), dHCM trophoblast layer side and dHCM reticular layer side. Representative images of H&E and Masson's trichrome staining of dHCM subcutaneously implanted on balb/c mice in three different time-points (4, 10 and 28 days) **(C)**. Where, * $p \leq 0.05$; *** $p \leq 0.001$ and; **** $p \leq 0.0001$.

IV-4. DISCUSSION

In this study, a natural biomaterial was obtained resulting from a decellularization protocol previous developed by us for the HCM alone [21]. dHCM was characterized for its composition, mechanical properties and *in vitro* and *in vivo* biocompatibility.

In order to avoid cell and host adverse response, a decellularized tissue should 1) lack nuclear material (stained with DAPI); 2) have no DNA fragments larger than 200 bp; and 3) present an amount

of dsDNA/mg of dry tissue less than 50 ng [4]. All these criteria were achieved by our process to obtain dHCM. During the decellularization process, the TL was removed being obtained a compact and easy to manipulate membrane. A combination of decellularization methodologies comprising mechanical, chemical, detergent and enzymatic techniques was used in this work. Although the use of SDS and Triton-X in tissue decellularization is associated with the removal of cellular components and nuclear remnants, it is also associated with collagen damage, disruption and removal of GAGs [4]. This was observed in our results and it is in accordance with previous reports, since there was a decrease in the quantity of soluble collagen [25] and sulfated GAGs [25–27] in dHCM and TL (both submitted to the decellularization process) when compared to HCM. However, insoluble collagen content increased (per mg of dry tissue) in dHCM when compared with HCM. In fact, the total collagen amount also follows this trend. This phenomenon has been previously reported [27–30]. Due to the removal of GAGs and other cellular proteins during the decellularization process, the amount of the remain collagen tends to increase per dry weight [27,30]. Overall, the obtained results suggest that our decellularization process was efficient in removing cells and DNA without substantial ECM damage. SDS-PAGE, dot blot and immunohistochemistry's tissue sections demonstrating the preservation of ECM proteins such as collagen type I, collagen type IV, fibronectin and laminin, corroborate the previous results. Altogether, it is demonstrated that the decellularization protocol used herein is adequate, since the exposure time to detergents is relatively short, and not too aggressive to induce major ECM disruption. The maintenance of ECM proteins in dHCM is of great importance since it maintains the specific cell microenvironment and can guide cell behavior [31], giving structural support to the tissue, and functional input to modulate cell performance and function [32]. In fact, other ECM-based membranes, such as SIS (porcine small intestinal submucosa) [33] and dHAM [34], are also composed by these proteins. However, dHAM also presents collagen type III and V that were not studied in this work.

The young's modulus represents the stiffness or resistance to elastic deformation under load of a biomaterial and the ultimate tensile strength characterizes the resistance of a material to fracture. Both the Young's modulus and the ultimate tensile strength are higher in dHCM when compared to HCM ($p < 0.0001$), as already demonstrated [29]. This may be explained because the ECM is composed by several polymerized proteins that aggregate through non-covalent bonds, creating a fibrous network. The cells bind to the ECM through weaker bonds, the focal adhesions [35]. So, if the decellularization process is not too aggressive to damage the ECM proteins, it is expected that the native tissue (with weaker bonds) show lower stiffness. Also the weaker cell-ECM bonds act locally as stress concentration or local structural defects, amplifying locally the stress field and leading to lower resistance to fracture of the native tissue

[36,37]. The Young's modulus of dHCM and ultimate tensile strength are both higher than SIS [38] but lower than acellular amnion membrane [39]. Nevertheless, the ability to suture dHCM was also indirectly demonstrated during our decellularization process, used in our case to identify the two sides of the dHCM.

The swelling behavior is an important parameter in the characterization of the dHCM being associated with the stability of the membrane in aqueous medium. Thus, to perform this study, culture medium and PBS were used to characterize the stability of dHCM during cell culture and storage, respectively. It was observed that after 30 min, the swelling behavior of dHCM stabilized. Thus, before each cell seeding stage, both membranes (dHCM and CTR) were immersed in culture medium overnight to ensure stable and comparable wetting of the membranes.

The cytocompatibility of dHCM was investigated using two human cell lines, of endothelial cells (EA.hy926) and fibroblasts (MRC-5). Commercial inserts were used as reference. SEM micrograph) and immunohistochemistry's tissue sections demonstrated that after the decellularization protocol, a membrane with two different surfaces (reticular layer side and trophoblast layer side) was obtained. Both sides of dHCM showed to be cytocompatible with the two human cell lines. Additionally, no differences were observed between dHCM trophoblast layer side and dHCM reticular layer side with the exception of day 3 that suggests a better performance of dHCM trophoblast layer side with both cell lines. On day 6, CTR condition outperformed both sides of dHCM. This could be associated with SDS-associated toxicity. Nevertheless, *in vivo* subcutaneous results refute this assumption. So, it is hypothesized that the different surface areas of commercial inserts (0.33 cm²) and dHCM inserts (0.95 cm²) might influence cells to behave and organize in different ways [40], resulting in a distinct metabolic activity pattern overtime between conditions. Moreover, corroborating this hypothesis, it is demonstrated that cellular metabolic activity increases significantly from day 3 to day 6 in both dHCM sides with both cell lines.

ECM scaffolds, as dHCM, are envisioned to help and accelerate the remodeling of injured tissues *in vivo* [41]. By its subcutaneous implantation in animal models, it is possible to obtain information about the elicited inflammatory response towards the biomaterial implanted and about its integration with the host tissue [42,43]. In summary, a healthy host response after biomaterial implantation is characterized by an acute pro-inflammatory phase mainly orchestrated by neutrophils, monocytes and macrophages, followed by an anti-inflammatory phase mediated by the transition from M1 to M2 macrophages. This response may end with fibroblast recruitment and collagen deposition that leads to a fibrous capsule formation [44–46]. So, a fibrous capsule formation surrounding the biomaterial is a common response after subcutaneous implantation [47–49]. Nevertheless, the formation of a fibrous capsule was observed

4 days after implantation, suggesting a rapid recovery of the pro-inflammatory phase associated with biomaterial implantation. This also demonstrates that dHCM final washes with PBS are sufficient and efficient since, it is free of DNase and detergents (such as SDS) associated with adverse immune responses *in vivo* [50]. Importantly, on day 28, it is possible to observe that dHCM membranes are being integrated by the host tissue revealing the beginning of a tissue regeneration stage. In contrast with SIS that is almost completely infiltrated by host cells after 1 week subcutaneously [49]. However, SIS has been associated with higher recurrent rates in hernia repairs [51–53] that may be associated with rapid degradation and integration. So, the longer time until integration by host tissue observed with dHCM might be a positive point, allowing a sustained tissue regeneration and eventually decreasing unwanted/adverse effects such as hernia recurrence. Moreover, in general, dHCM and dHAM have a similar behavior *in vivo* [34]. Altogether, these results support the hypothesis that dHCM may be used as a biomaterial for promoting tissue regeneration in case of hernias, barrier defects, wounds, burns and/or skin defects.

IV-5. CONCLUSIONS

In this work, the dHCM was fully characterized, for the first time, for the presence and distribution of ECM components, mechanical properties and *in vivo* and *in vitro* behavior. It was demonstrated that the dHCM is in accordance with the established criteria for decellularized tissues to avoid cell and host adverse reactions [4]. As SIS and dHAM, dHCM preserved some important ECM proteins such as collagen type I, collagen type IV, fibronectin and laminin and showed to be biocompatible *in vitro* and *in vivo* in subcutaneous implantation. Nevertheless, as a differentiator characteristic, dHCM has two different surfaces (reticular layer side and trophoblast layer side) that have different composition and topography. These results, together with the ability to suture dHCM and its mechanical properties, support the hypothesis of dHCM to be used as a biomaterial for tissue regeneration applications, particularly when a membrane is needed to separate tissues, organs or other biologic compartments.

IV-6. FUNDING

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IV-7. ACKNOWLEDGMENTS

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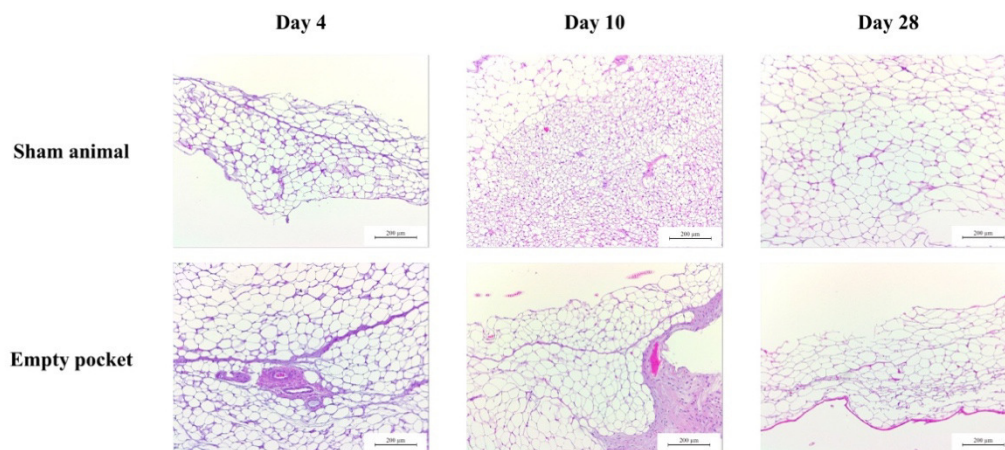
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IV-9. SUPPLEMENTARY MATERIAL

Supplementary Figure IV-1 Representative images of H&E staining of control animals used



in the subcutaneous assay performed on BALB/c mice at three different time-points (4, 10 and 28 days). In sham animals only the surgery was performed (without dHCM implantation). Empty pockets were performed on the contralateral side of animals implanted with dHCM.

SECTION 4

DECELLULARIZED HUMAN CHORION MEMBRANE (DHCM) APPLICATIONS

Chapter V

A functional Blood-Brain Barrier *in vitro* model using decellularized human chorion membrane

Chapter V

Decellularized human chorion membrane as a substrate to create a BBB *in vitro* model[†]

ABSTRACT

The creation of functional blood-brain barrier (BBB) *in vitro* models is of great interest for drug screening, to study diseases' mechanisms, and to create valid alternatives to animal testing. In this work, we demonstrated the suitability of the decellularized human chorion membrane (dHCM) to create an *in vitro* model of the BBB. For that, cerebral endothelial (bEnd.3) and astrocytic (C8-D1A) cell lines were used. Our results demonstrated that dHCM can mimic the BBB basement membrane and that the transendothelial electrical resistance (TEER) values were higher in our dHCM-based BBB *in vitro* model than the ones described in literature. Moreover, we determined the apparent permeability coefficient (P_{app}) of three different transport routes across BBB (membrane diffusion, solute carrier, and receptor-mediated) by using caffeine, glucose, and transferrin, respectively. The expression of glucose (GLUT1) and transferrin receptors by bEnd.3 cells was also demonstrated. In this work, we demonstrated that dHCM is a suitable substrate to create an *in vitro* model of BBB.

[†] This chapter is based on the following publication:

Frazão, L. P.; Vieira de Castro, J.; Nogueira-Silva, C.; Neves, N.M. Decellularized human chorion membrane as a substrate to create a BBB *in vitro* model (submitted). Associated with the international patent application WO/2021/005531.

V-1. INTRODUCTION

The blood-brain barrier (BBB) is a biological barrier that occurs along the capillaries in the brain being responsible to supply nutrients and oxygen to the brain, to maintain water and electrolyte balance in the brain interstitial space, and to protect neurons from potentially harmful substances present in the blood [1,2]. The BBB is mainly composed by specific endothelial cells (with more mitochondria and less pinocytic vesicles when compared with peripheral endothelial cells) that communicate and are influenced by several different cells such as pericytes, astrocytes, neurons, and microglia, which compose the neurovascular unit (NVU) [2–5]. One of the main concerns about the BBB is the transportation of drugs [6,7]. As a biological barrier, the BBB has a restrictive nature that is mainly influenced by tight junctions within endothelial cells, which regulate paracellular permeability and specific cellular receptors and transporters limiting molecule transportation across the BBB [5,8,9]. So, the creation of functional BBB *in vitro* models mimicking the *in vivo* scenario is of great interest not only for drug screening and for the study of diseases' mechanisms but also to create alternatives, with a strong predictive power, to animal testing [10–14].

There are several established BBB *in vitro* models based on mono-cultures, co-cultures (in direct or indirect contact), tri-cultures or multi-cultures of endothelial cells with other NVU cells as reviewed by Wolff, *et al.* [3]. The origin of the cells used differs from pig, bovine, rat, mouse, and human. Moreover, the cells can be seeded either in static, dynamic, or microfluidic models [3]. Unfortunately, there is no consensual model or cell type defined for BBB studies *in vitro*, raising difficulties in the comparison of results between groups [1,3,15].

It is of great interest to mimic the extracellular matrix (ECM) *in vitro* since it is important for vessel development, stability, and barrier function [16–19]. Thus, with this work, we intend to create a functional, readily available, and convenient to use BBB *in vitro* model using the decellularized human chorion membrane (dHCM) to mimic the BBB basement membrane. The use of dHCM is novel in this application since previous studies only used coatings and/or formulations with ECM proteins [20–23]. The main advantage of using dHCM relies in the capacity that the decellularized membrane has to maintain the main proteins of the ECM and its native architecture [24]. Moreover, dHCM is composed by collagen type I, collagen type IV, fibronectin, and laminin [24], proteins that also compose the BBB basement membrane [15,25].

To create the BBB *in vitro* model using dHCM, mice cell lines of brain capillary endothelial cells (bEnd.3) and astrocytes (C8-D1A) were used. Additionally, transendothelial electrical resistance (TEER) measurements and functional permeability assays were performed to validate the model.

V-2. MATERIALS AND METHODS

V-2.1. Decellularized human chorion membrane (dHCM)

dHCM were obtained as previously described by us [24,26]. Placenta collection and processing followed a protocol approved by the ethical committee of the Hospital de Braga and of the University of Minho (CESH 030/2016, SECVS 136/2015, respectively) and followed all the international best practices for research with human biological samples, including a signed informed consent from the donors.

V-2.2. Cell culture and metabolic activity

Mouse brain endothelial cell line bEnd.3 at passages 27 to 33 and mouse astrocyte cell line C8-D1A at passages 3 to 14 were cultured in complete medium (DMEM high glucose (#D5648, Sigma-Aldrich) with 1.5 mg/mL sodium bicarbonate (#S586, Sigma-Aldrich), 1% sodium pyruvate (#11360039, Gibco), 10% fetal bovine serum (FBS; #A3160801, Thermo Fisher Scientific) and 1% Penicillin/Streptomycin (#15240062, Thermo Fisher Scientific)) in T150 flasks at 37 °C in a humidifier incubator with 5 % CO₂. When the cells reach 80% of confluence, they were trypsinized and transferred to inserts and culture plates. dHCM were mounted in cell crown inserts for 24-well plates (#Z742380-12EA, Sigma) (Figure V-1). Inserts were immersed in culture medium overnight before cell seeding. C8-D1A cells were seeded in adherent 24-well plates with a density of 20 x 10³ cells/cm². bEnd.3 cells were seeded on the upper part of the inserts with a density of 20 x 10³ cells/cm² and the insert was then placed in a 24-well plate. For monocultures, an insert without cells was added to the seeded wells. For co-cultures, the cells were seeded at the same time. After 3 h, the inserts were moved to the wells seeded with C8-D1A cells and culture medium was added (Figure V-1). Metabolic activity was evaluated by MTS assay (CellTiter 96 AQueous One Solution, Promega) according to the manufacturer's instructions.

V-2.3. Fluorescence imaging

Phalloidin/DAPI staining was performed in fresh tissue, fixed with 10% formalin for, at least 24 h at RT. Firstly, samples were incubated with 1:200 Phalloidin-TRITC, (#P1951, Sigma-Aldrich), at RT for 1 h. Then, DAPI (#40009, VWR), at 1:100000 was added for 1-5 min at RT. Samples were observed under a fluorescence microscope with a coupled camera (Axio Imager Z1m, Zeiss). Images were analyzed with Zeiss Zen microscope software.

V-2.4. TEER measurements

Transendothelial electrical resistance (TEER) was measured using a Millicell® ERS-2 Electrical System (MERS00002, Millipore) in accordance with manufacturer's instructions. Each condition was performed in triplicate at two different time-points (days 3 and 7), and five TEER measures were done per sample. At least three independent assays were performed. TEER values were calculated by two different methods already described [27,28]. The first method consisted in the calculation of TEER values of the bEnd.3 monolayer. For that, a blank value (resistance of the dHCM trophoblast layer side without cells) was subtracted from the total resistance of mono- and co-culture conditions. Then, the values were normalized for the surface area [28]. The second method aimed to obtain TEER values of the system (bEnd.3 monolayer with dHCM), where, the resistance value was directly normalized by the surface area [27].

V-2.5. Permeability assays

Permeability assays were performed in triplicate at two different time-points (days 3 and 7). At least three independent assays were performed. In general, the inserts were transferred to a new receiver 24-well plate with 1 mL of fresh culture medium. Then, 400 µL of working solution was added to the upper side of the insert. The apparent permeability coefficient (P_{app}) was calculated according to the following equation:

$$\text{Apparent Permeability Coefficient } (P_{app}) = ((V_{up} / (S_A * t)) * (C_R * C_{up}))$$

Where: V_{up} is the volume added to the upper side of the insert (400 µl); S_A is the surface area of the insert (0.95 cm²); t is the time in s; C_R is the concentration in the receiver plate; and C_{up} is the initial concentration added to the upper side of the insert.

V-2.5.1. FITC-dextran

Ten µg/mL of 70 kDa FITC-dextran (#46944 and #46945, Sigma-Aldrich, respectively) in culture medium were used as working solutions and were incubated for 2 h at 37 °C in a humidifier incubator with 5% CO₂. One hundred µL of the collected medium from the receiver plate were transferred to an opaque 96-well plate and fluorescence was read using an excitation of 485 nm and emission of 535 nm, in a microplate reader (SynergyHT, Biotek).

V-2.5.2. Caffeine

Seventy-five µg/mL of Caffeine (#C0750, Sigma-Aldrich) in culture medium was used as working solution and incubated for 3 h at 37 °C in a humidifier incubator with 5% CO₂. Caffeine ELISA kit (#DEIA6842, Creative Diagnostics) was used to quantify the caffeine in the collected medium, in accordance with manufacturer's instructions.

V-2.5.3. Glucose

Four (4.41) mg/mL of Glucose (#G8270, Sigma-Aldrich) in culture medium was used as working solution and incubated for 3 h at 37 °C in a humidifier incubator with 5% CO₂. Glucose Assay Kit (#ab102517, abcam) was used to quantify glucose in the collected medium, in accordance with manufacturer's instructions.

V-2.6. Transferrin uptake assay

Transferrin uptake assay was performed in triplicate at two different time-points (days 3 and 7). At least three independent assays were performed. Firstly, cells were starved by transferring inserts for a new receiving plate with 1 mL of culture medium without FBS and 400 µL of the same starving medium were added to the upper side of the insert. After 1h of incubation at 37 °C in a humidifier incubator with 5 % CO₂, the medium in the upper chamber of the insert was removed and replaced by 400 µL of a working solution of 25 µg/mL of biotinylated transferrin (#T3915, Sigma-Aldrich) in starving medium. Cells were incubated for 30 min and then fixed with 10% formalin for at least 24 h at RT. After three washes with PBS, biotin signal was revealed using streptavidin 1:500 (#S11227, Thermo Fisher Scientific). Samples

were analyzed under a fluorescence microscope with a coupled camera (Axio Imager Z1m, Zeiss). Images were analyzed with Zeiss Zen microscope software.

V-2.7. Immunofluorescence

Immunofluorescence was performed in fresh samples, fixed with 10% formalin for, at least, 24 h at RT. Briefly, after a permeabilization with 0.5% Triton X-100 (#A16046-0100, Alfa Aesar) for 10 min at RT and a blockage with 5% BSA (#A2153, Sigma-Aldrich) for 1 h at RT, samples were incubated with rabbit anti-GLUT1 1:50 (#ab15309, abcam), and rabbit anti-Transferrin Receptor 5 µg/ml (#ab84036, abcam) antibodies, overnight at 4 °C. Donkey anti-rabbit 594 1:500 (#A21207, alfa gene) was used as secondary antibody and was incubated for 1 h at RT. Samples were counterstained with DAPI (#40009, VWR) at 1:10000. A fluorescence microscope with a coupled camera (Axio Imager Z1m, Zeiss) was used to obtain images that were further analyzed with the Zeiss Zen microscope software.

V-2.8. Statistical analysis

Statistical analysis was performed using Graph Pad Prism 7. Shapiro-Wilk test was used to assess the data normality. As data followed a normal distribution, a parametric test was used, namely two-way ANOVA test followed by Turkey's multiple comparisons test. A $p < 0.05$ was considered statistically significant in the analysis of the results.

V-3. RESULTS

V-3.1. dHCM trophoblast layer side is more suitable for a BBB *in vitro* model

The metabolic activity of bEnd.3 and C8-D1A cells lines was analyzed when seeded with dHCM. As demonstrated in Figure V-1, bEnd.3 cells were seeded in direct contact with dHCM whereas C8-D1A cells were seeded on the well, being in indirect contact with dHCM. As previously described by us [24], dHCM has two sides with different composition and topography (reticular and trophoblast layer sides). The main objective of this assay was to demonstrate the cytocompatibility of dHCM with these cells lines and also to determine which side would be better to proceed the studies with. When dHCM side is

presented, it corresponds to the dHCM side that is in contact with the upper chamber of the insert (as shown in Figure V-1).

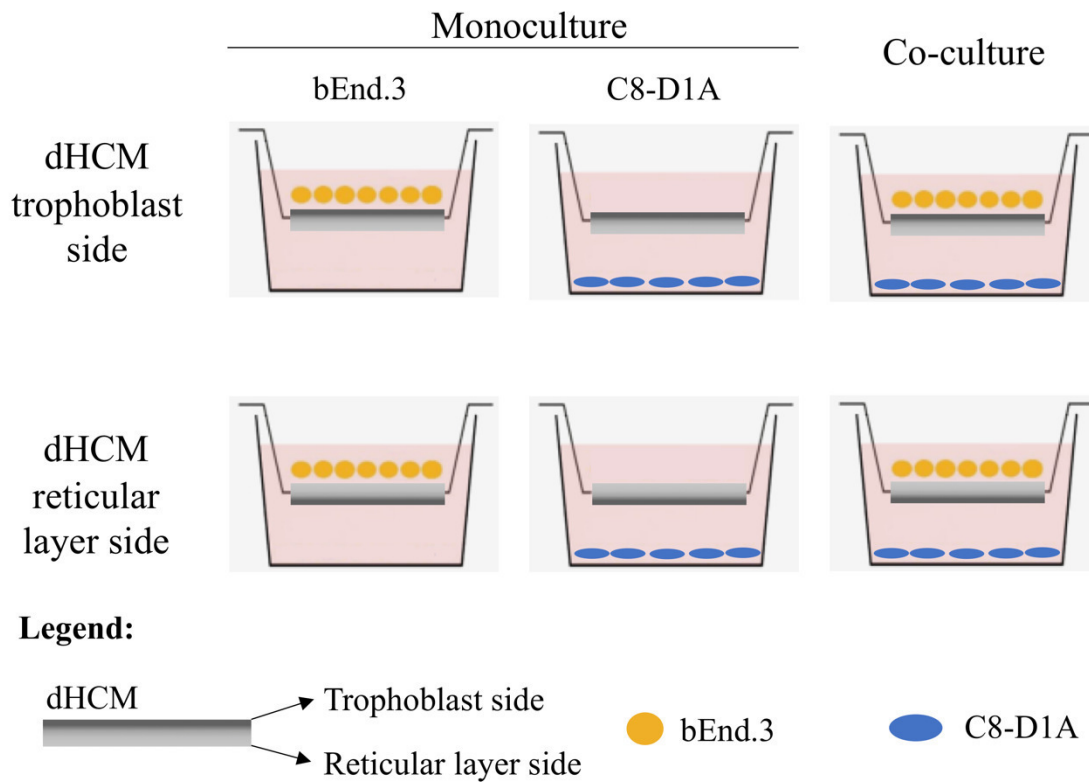


Figure V-1 Schematic representation of bEnd3 and C8-D1A cells seeding on both sides of dHCM in mono and co-culture conditions

For bEnd3 cell line both in mono- and co-culture (Figure V-2A), dHCM trophoblast layer side was associated with a slightly higher metabolic activity when compared with dHCM reticular layer side. Nevertheless, a statistically significant difference between dHCM sides was only observed on day 7 in monoculture condition ($p = 0.0052$). Regarding C8-D1A cell line, the same tendency was observed (Figure V-2B). However, no statistically significant differences were observed between both dHCM's sides. bEnd.3 metabolic activity was relatively stable overtime (Figure V-2A). In contrast, C8-D1A metabolic activity increased overtime in both dHCM trophoblast layer side ($p < 0.0001$ (monoculture and co-culture)) and dHCM reticular layer side ($p < 0.0001$ (monoculture)) (Figure V-2B). The Phalloidin/DAPI staining corroborated these results (Figure V-2C and 2D) and showed that a monolayer of bEnd.3 cells was

achieved on day 3 in both dHCM sides (Figure V-2C). An increase in C8-D1A cells confluency was observed from day 3 to day 7 in both conditions (Figure V-2D). Taking into account these results, future assays were performed by culturing bEnd.3 cells in the trophoblast layer side of the dHCM in the upper side of the insert either in monoculture or in co-culture with C8-D1A cells cultured in the bottom of the well (non-contact).

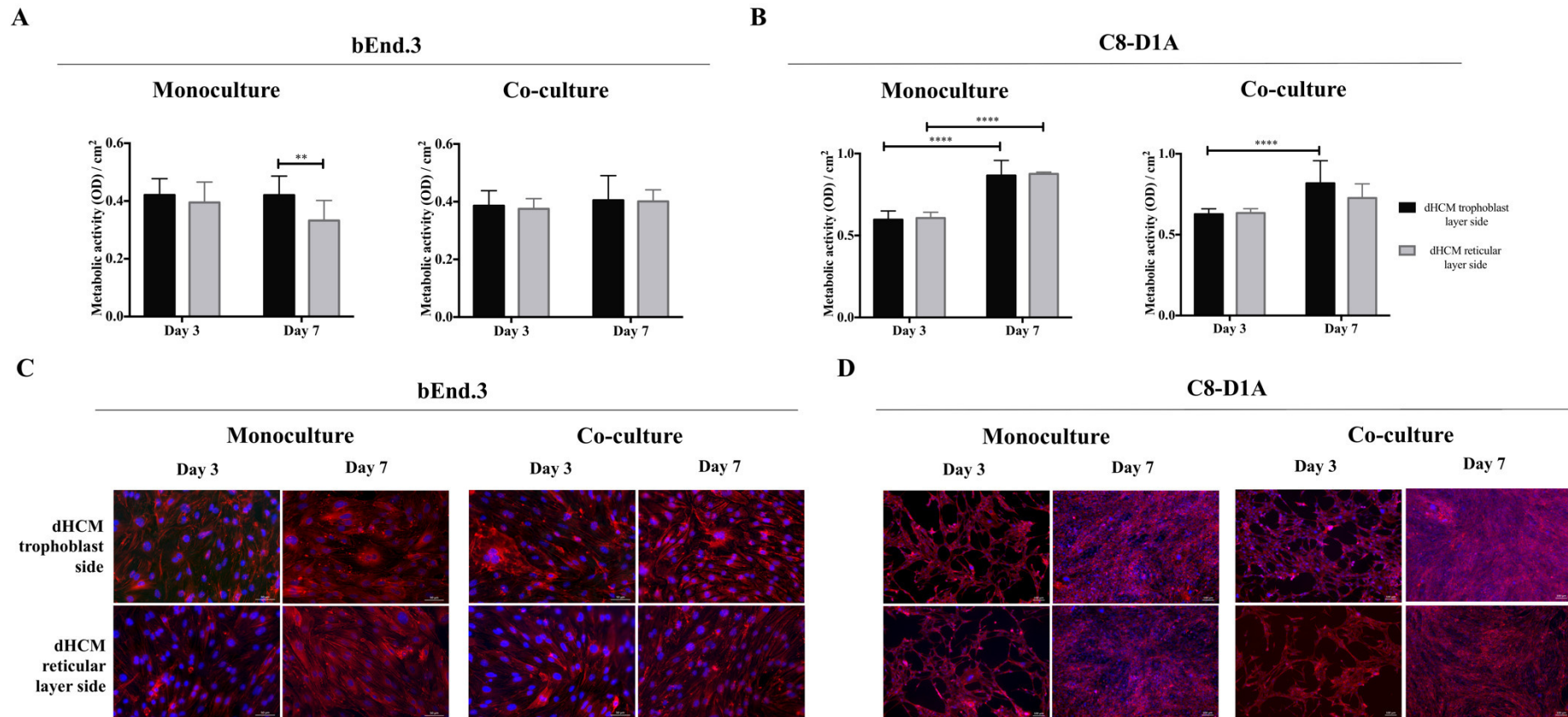


Figure V-2 The suitability of dHCM for a BBB *in vitro* model. Metabolic activity (**A - B**) and phalloidin/DAPI staining (**C - D**) of bEnd.3 (**A and C**) and C8-D1A (**B and D**) cells in dHCM trophoblast side and dHCM reticular layer side in both mono and co-culture conditions at days 3 and 7 of culture.

V-3.2. BBB model validation

To validate our BBB model, TEER measurements (Figure V-3A-B) and diffusion assays (Figure V-3C) were performed. As expected, TEER values for bEnd.3 monolayer and for the system (bEnd.3 monolayer with dHCM) behaved in the same way (Figure V-3A-B). In general, monoculture condition (mean $20.47 \pm 0.40 \Omega\text{cm}^2$ for bEnd.3 cells in monolayer (Figure V-3A) and mean $108.00 \pm 11.77 \Omega\text{cm}^2$ for the system (Figure V-3B)) was associated with slightly lower TEER values when compared to co-culture condition (mean $32.46 \pm 12.78 \Omega\text{cm}^2$ for bEnd.3 cells in monolayer (Figure V-3A) and mean $118.70 \pm 23.07 \Omega\text{cm}^2$ for the system (Figure V-3B)). Nevertheless, no statistically significant differences were observed between mono- and co-culture conditions. On day 7, a TEER decrease was observed in all conditions, achieving a statistically significant difference in system values in co-culture ($p = 0.0091$; Figure V-3B).

To further assess the integrity of our model, diffusion assays with FITC-dextran with 70 kDa were performed (Figure V-3C). In general, FITC-dextran diffusion was stable overtime and no statistically significant differences were observed between mono- and co-culture overtime. The P_{app} of 70 kDa FITC-dextran was $7.09 \pm 0.32 \times 10^{-6} \text{ cm/s}$ for monocultures and $7.09 \pm 0.42 \times 10^{-6} \text{ cm/s}$ for co-cultures.

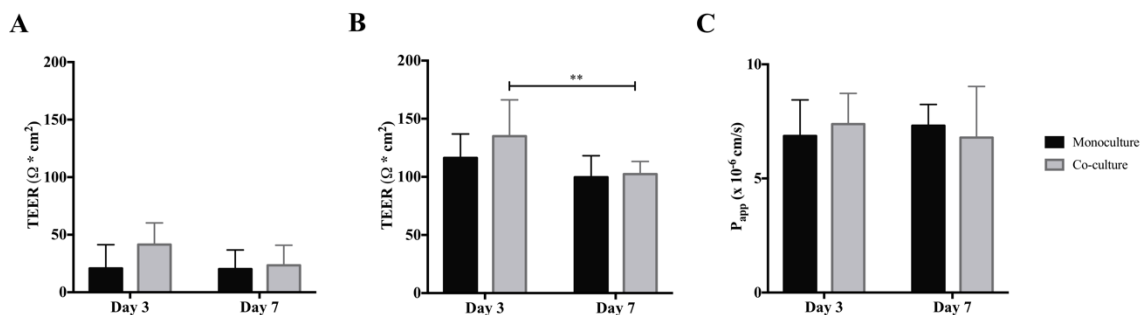


Figure V-3 BBB model validation. TEER values were obtained for bEnd.3 monolayer (A) and for the system that is composed by bEnd.3 monolayer and dHCM (B), both in mono and co-culture conditions. Diffusion assays with and 70 kDa FITC-dextran (C) were also performed for mono and co-cultures, at two different time-points (days 3 and 7). Where ** $p \leq 0.01$.

V-3.3. Functional BBB permeability

For functional permeability assays, molecules that are known to cross the BBB (caffeine, glucose, and transferrin) were used. For caffeine (Figure V-4A), P_{app} was higher in monoculture condition when compared with co-cultures on day 3 ($p = 0.0043$) and day 7 ($p = 0.0187$). However, P_{app} of caffeine was stable overtime, being $7.42 \pm 0.03 \times 10^{-6}$ cm/s in monoculture and $6.99 \pm 0.01 \times 10^{-6}$ cm/s for co-cultures (Figure V-4A). Regarding glucose, the same tendency, with slightly higher P_{app} values for monocultures was observed. However, no statistically significant differences were found (Figure V-4B). The P_{app} of glucose was $2.40 \pm 0.23 \times 10^{-5}$ cm/s for monocultures and $2.16 \pm 0.28 \times 10^{-5}$ cm/s for co-cultures. Finally, transferrin uptake was evaluated (Figure V-4C). On day 3, transferrin uptake appeared to be higher than on day 7 in both mono- and co-culture conditions. However, no differences were observed between mono- and co-cultures conditions.

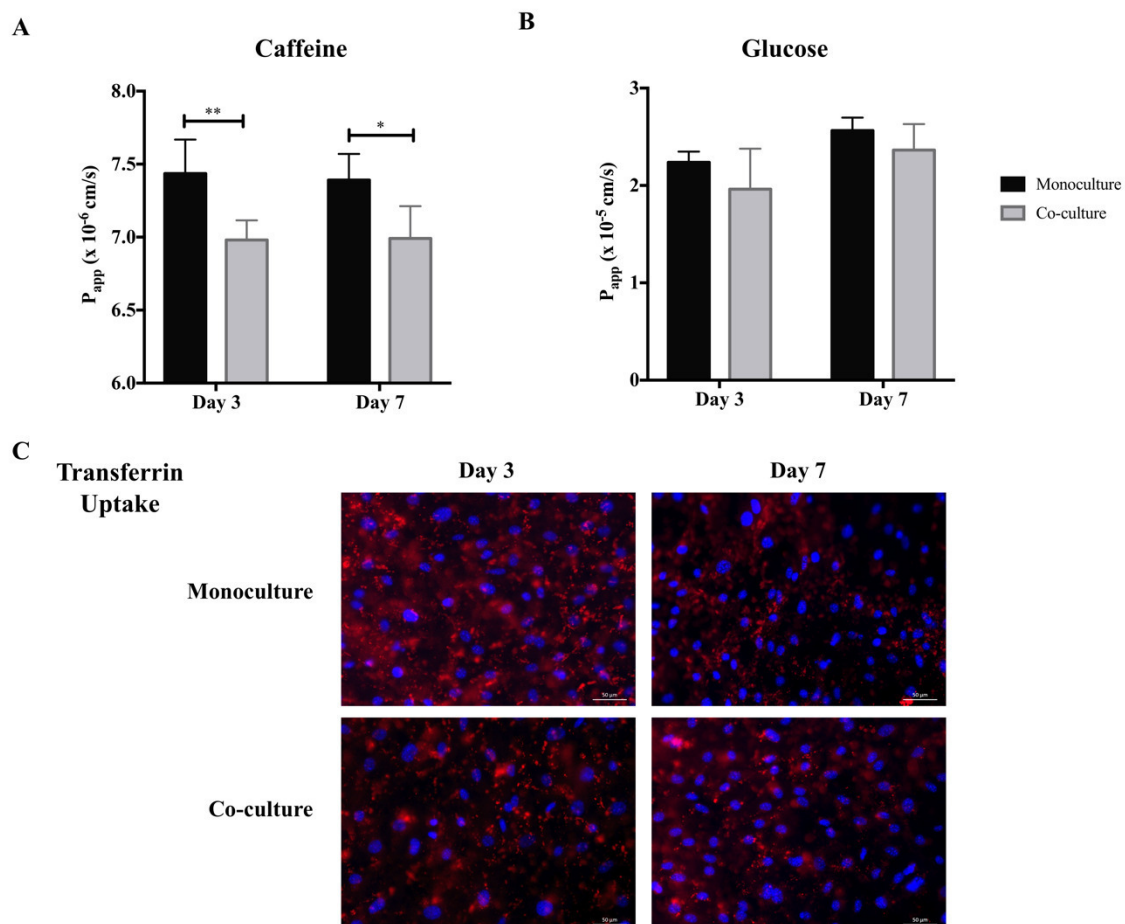


Figure V-4 Functional BBB permeability. Different molecules were used to exemplify the different transport systems at the BBB. Caffeine was used for membrane diffusion **(A)**, glucose for carrier-mediated transport **(B)**, and transferrin for receptor-mediated transport **(C)**. Where * $p \leq 0.05$; ** $p \leq 0.01$.

Glucose and transferrin cross the BBB through a carrier and a transporter, respectively [9]. So, to demonstrate the functional transportation of these molecules across the BBB, both GLUT1 and transferrin receptor expression were assessed. As demonstrated on Figure V-5A, GLUT1 expression was very similar between mono and co-culture conditions and was stable overtime. The same was observed for transferrin receptor (Figure V-5B). These results corroborate the previous ones of the P_{app} of glucose and transferrin uptake (Figure V-4B and 4C).

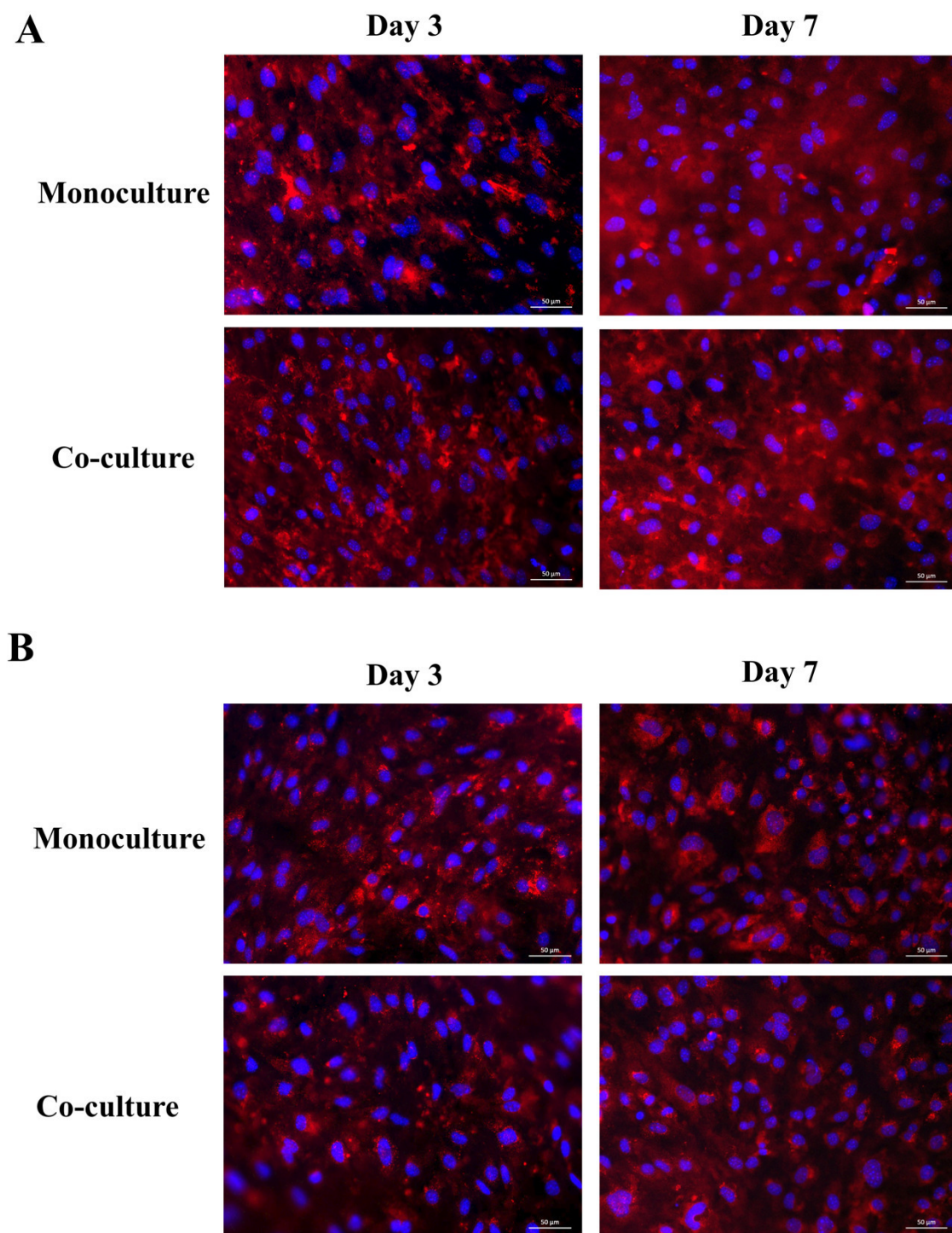


Figure V-5 GLUT-1 and transferrin receptor expression. GLUT1 **(A)** and transferrin receptor **(B)** expression were assessed over time (days 3 and 7) in both conditions (mono and co-culture) by immunofluorescence.

V-4. DISCUSSION

In this work, we demonstrated that dHCM may be used as a substrate to create *in vitro* models, specifically a model of BBB. We had previously demonstrated that while dHCM reticular layer side was mainly composed by collagen type I, the dHCM trophoblast side was composed by collagen type I, collagen type IV, fibronectin and laminin [24], proteins that also compose the BBB basement membrane [15,25]. So, bEnd.3 cells seeded in the dHCM trophoblast side was the chosen condition to the further assays.

Regarding TEER assays, the values obtained in this work are far from the ones described *in vivo* ($8000 \Omega\text{cm}^2$) [29]. Nevertheless, they are in accordance with TEER values previously described for *in vitro* studies using bEnd.3 monolayer in both monoculture and co-culture with C8-D1A cells. In fact, in general, our model presents higher TEER values than the ones previously described for these cell types [27,28]. Regarding TEER values of bEnd.3 monolayer, the mono- and co-culture values of our model ($20.47 \pm 0.40 \Omega\text{cm}^2$ and $32.46 \pm 12.78 \Omega\text{cm}^2$, respectively) are higher than the previously described values using fibronectin coated transwells ($10 - 15 \Omega\text{cm}^2$ for monocultures and $20 - 25 \Omega\text{cm}^2$ for co-cultures) [28]. Considering the TEER values for the system (bEnd.3 monolayer with dHCM), our model presents higher values ($108.00 \pm 11.77 \Omega\text{cm}^2$ for monoculture and $118.70 \pm 23.07 \Omega\text{cm}^2$ for co-culture) than the ones using collagen coated transwells ($\sim 96 \Omega\text{cm}^2$ for monoculture and $102.4 \Omega\text{cm}^2$ for co-culture) [27]. Other TEER values reported in literature using endhom chambers [30], cannot be compared with ours, since the methodology for obtaining them was different. Several studies used FITC-dextran (with different sizes) to assess the paracellular permeability of a BBB model [27,28,31]. In the present work, FITC-dextran with 70 kDa was used. Our model had a higher permeability ($7.09 \pm 0.32 \times 10^{-6} \text{cm/s}$ (monoculture), $7.09 \pm 0.42 \times 10^{-6} \text{cm/s}$ (co-culture)) than the ones described for non-static mono- and co-cultures with the same cell types (approximately $1 \times 10^{-6} \text{cm/s}$) [28] and for *in vivo* (approximately $0.15 \times 10^{-6} \text{cm/s}$) [32].

To assess the functionality of this dHCM based BBB *in vitro* model, different molecules were used to validate the efficacy in modeling the transportation across the BBB. Caffeine was used as an example of membrane transport, glucose as a carrier-mediated transport (solute carrier), and transferrin as an example of receptor-mediated transport [33]. For the first time, in this work, the P_{app} of caffeine using bEnd.3 and C8-D1A cells was obtained (around $7 \times 10^{-6} \text{cm/s}$). These values are similar to the ones obtained with an artificial membrane ($0 - 8 \times 10^{-6} \text{cm/s}$) [34], lower than the ones obtained using porcine

cells (16×10^{-6} cm/s) [35], but higher than the ones achieved with human cells (2.1×10^{-5} cm/s) [36]. Although we demonstrate in our model, one of the lowest P_{app} obtained *in vitro* for caffeine, *in vivo* permeability to caffeine is around 1×10^{-4} cm/s [37]. Regarding glucose transport, P_{app} obtained in our *in vitro* BBB model ($2.40 \pm 0.23 \times 10^{-5}$ cm/s for monocultures and $2.16 \pm 0.28 \times 10^{-5}$ cm/s for co-cultures) are slightly higher but in the same order of magnitude than the permeability values described *in situ* (1×10^{-5}) [15]. Transferrin uptake by bEnd.3 cells was also demonstrated. This is important for drug delivery since transferrin receptor expression is limited to the brain endothelium, being absent in peripheral endothelium. So, several drug delivery strategies are focused in this pathway [38,39]. Moreover, we also demonstrate the expression of GLUT1 and transferrin receptor by bEnd3 cells. These results are promising since they may suggest that the transportation of glucose and transferrin is mediated by their receptors, mimicking the *in vivo* condition.

In general, no significant differences were observed between mono- and co-culture. In fact, it was described that hCMEC/D3 (human cerebral endothelial cell line) respond better to astrocyte contact (as occurs *in vivo*) rather than the soluble factors produced by astrocytes [40]. Our results suggest that this may also happen with bEnd.3 cells.

V-5. CONCLUSIONS

In this work, the dHCM was used as substrate to create an *in vitro* model, by mimicking the BBB basement membrane. The TEER values of our dHCM-based BBB *in vitro* model were higher than the ones previously described. Moreover, three different transport routes across BBB (membrane diffusion, solute carrier, and receptor-mediated) seemed to be functional. In conclusion, our work clearly demonstrates that dHCM is a suitable substrate to create an *in vitro* model of the BBB.

V-6. FUNDING

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V-7. ACKNOWLEDGMENTS

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Chapter VI

The decellularized human chorion membrane as a vascular graft

Chapter VI

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ABSTRACT

The increase of both arterial occlusive diseases and coronary heart diseases leads to a higher demand for small diameter vascular grafts (< 6 mm). The gold standard for small diameter vessel replacement is the use of autologous veins. Nevertheless, up to 30% of these patients need to use vascular grafts. Although synthetic polymers have been successfully used for the replacement of large diameter vascular grafts (> 6 mm), they are associated with thrombosis, intimal hyperplasia, calcification and chronic inflammation when used as small diameter vascular grafts. So, natural materials have been studied for this application. In this study, a decellularized human chorion membrane (dHCM) vascular graft with 3-4 mm diameter was created. Herein, the biocompatibility of dHCM with endothelial cells was demonstrated *in vitro* and *ex ovo*. Blood biocompatibility of dHCM was also shown by studying plasma protein adsorption, platelet adhesion and activation and its hemolytic potential. Furthermore, dHCM antibacterial properties against *S. aureus* were also studied. In summary, the dHCM reticular layer side presented all the needed characteristics to be used in the inner side of a vascular graft. Additionally, the mechanical properties of dHCM tubular construct were studied, being similar to the ones of the saphenous vein, the gold standard for autologous small diameter vessel replacement.

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VI-1. INTRODUCTION

Cardiovascular disease is the leading cause of death worldwide, being associated with a high morbidity and mortality [1–3]. The increase of both arterial occlusive diseases and coronary heart diseases leads to a higher demand for small diameter vascular grafts (< 6 mm). The gold standard for small diameter vessel replacement is the use of autologous veins, such as the saphenous and umbilical veins, the internal thoracic and the radial artery [1,4,5]. However, due to the limited availability of healthy autologous vessels (associated with patients age and consequently blood vessel quality), there is a clinical demand for functional and long-term testing small-caliber blood vessels grafts [1,4,6,7]. Although synthetic polymers have been successfully used for the replacement of large diameter vascular grafts (> 6 mm), they are associated with thrombosis, intimal hyperplasia, calcification and chronic inflammation when used as small diameter vascular grafts [1,8]. Moreover, synthetic polymers do not have the capacity to grow overtime [9,10]. Natural materials have been investigated for this application. In general, they present excellent biocompatibility, but insufficient mechanical properties, being associated with vascular graft dilation and aneurysms [1]. Electrospun silk fibroin tubular constructs have been widely studied for the replacement of small diameter vascular grafts [11–15]. Other natural materials, such as collagen, elastin, hyaluronic acid, fibrin, and decellularized matrices have been also studied, as recently reviewed [9]. Additionally, materials derived from the human placenta have been also explored for this application. These studies used decellularized vessels from the human chorion plate [16,17], rolled decellularized amnion membranes [18,19], decellularized umbilical veins [20] and placenta extracellular matrix [21].

The decellularized human chorion membrane (dHCM) is a natural resource previously described by our group [22,23]. In contrast with decellularized vessels obtained from the human chorion plate [16,17,24], the dHCM allows the creation of tubes with different diameters. An ideal small diameter vascular graft should have good biocompatibility, hemocompatibility, infection resistance, off-the-shelf availability and appropriate mechanical properties to support the continuous stresses associated to blood circulation and to allow tissue regeneration *in vivo* [6,25,26]. Herein, we aim to study, for the first time, the suitability of dHCM as the basis to produce small diameter vascular grafts. For that, endothelial cell biocompatibility and *ex ovo* cell recruitment were both studied. Moreover, blood compatibility and resistance to *S. aureus* infection were assessed. Finally, dHCM was rolled onto a tube with the intended diameter and its mechanical properties were studied.

VI-2. MATERIALS AND METHODS

VI-2.1. Decellularized human chorion membrane (dHCM)

dHCM were obtained as previously described [22,23]. Briefly, placentas were collected and processed following a protocol approved by the ethical committee of the Hospital de Braga and of the University of Minho (CESH 030/2016, SECVS 136/2015, respectively). The chorion membrane was removed and stored at -80 °C. The decellularization of the chorion membrane involved a combination of detergents (0.5% and 0.1% SDS (#MB18101, NZYTech) and 1% Triton X-100 (#A16046, Thermo Fisher Scientific) with scrapping. DNase (#A3778, PanReac AppliChem ITW Reagents) was also used. The different sides of dHCM, trophoblast layer side and reticular layer side were compared.

VI-2.2. Tube formation assay and reendothelization assay

Human umbilical vein endothelial cells (HUVECs) were culture in medium 200 (#M-200-500, Gibco) supplemented with Large vessels endothelial supplement (LVES, #A14608, Gibco) in T75 and T150 flasks at 37°C in a humidifier incubator with 5% CO₂. When the cells reach 80% of confluence, they were trypsinized and transferred to inserts and culture plates. dHCM were mounted in cell crown inserts for 24-well plates (#Z742380-12EA, Sigma). The bottom of well of 48-well plates coated with 50 µL/cm² Matrigel (#354234, Enzifarma) were used as controls (in accordance with manufacturer's instructions) for the tube formation assay while tissue culture coverslips (#83.184.002, Sarstedt) were used as controls for the reendothelization assay. Inserts were immersed in culture medium overnight before cell seeding. HUVECs were seeded with a density of 25 x 10³ cells/cm². For the tube formation assay, the culture medium was collected and stored at -20°C and cells were fixed using 10% formalin (#5701, Thermo Fisher Scientific) after 4, 8, and 12 h of the seeding. For the reendothelization assay, cells were fixed using 10% formalin (#5701, Thermo Fisher Scientific) after 48 h. The represented dHCM side corresponds to the membrane side in direct contact with the cells. Three independent assays were performed.

VI-2.3. Quantification of angiopoietin 2

For the quantification of human angiopoietin 2 (ANG2), an ELISA kit (#ab99971, abcam) was used according to manufacturers' instructions. ANG2 values of all conditions were normalized to the level of ANG2 present in the culture medium alone.

VI-2.4. Chick chorioallantoic membrane (CAM) assay

For CAM assay, white fertilized eggs were incubated for 3 days at 37°C. Then, a window was opened into the eggshell to evaluate embryo viability. After 7 days (day 10 of the assay), dHCM were implanted on the CAM. The represented dHCM side corresponds to the dHCM in direct contact with CAM. Implanted eggs were returned to the incubator at 37 °C. At day 17, embryos were sacrificed by freezing at -80 °C for 10 min. After fixation with 4 % formalin, ex-ovo images were captured for each condition (CTR (without membrane), dHCM reticular layer side and dHCM trophoblast layer side) in both sides (chorionic and allantoic epithelium). At least 7 eggs were analyzed per condition (CTR (without membrane), dHCM reticular layer side and dHCM trophoblast layer side). The macroscopic evaluation of the angiogenic response was carried out as previously described [27]. Moreover, an automated angiogenesis quantification was performed using the Vessel Analysis plugin with Image J, where the measurements of vascular density and vascular length density are expressed as % area.

VI-2.5. Platelet adhesion assay

Blood (31.5 mL) was collected via venipuncture from healthy human volunteers in BD Vacutainer tubes (#367704, BD Diagnostics – Prenalytical Systems) containing sodium citrate. Platelet isolation was performed according to the abcam protocol. Briefly, platelet rich plasma (PRP) was obtained by centrifuging the collected blood at 200 *g* for 20 min at RT. Then, the PRP was mixed with HEP buffer 1:1 ratio (v/v) with 1 μ M prostaglandin E1 (P5515, Sigma-Aldrich) and centrifuged at 100 *g* for 18 min at RT. The supernatant was transferred to a new plastic tube and centrifuged at 800 *g* for 18 min. The pellet was then washed twice with Platelet Wash Buffer (without resuspension). The platelet concentrate was resuspended in 20 mL of Tyrode's buffer containing 5 mM glucose (#G8270, Sigma-Aldrich) and 3% BSA (#A2153, Sigma-Aldrich). All centrifugation steps were performed with no brake applied. As previously described, dHCM were mounted in cell crown inserts (#Z742380-12EA, Sigma). The represented dHCM

side corresponds to the membrane side in direct contact with the cells. dHCM and glass surfaces (used as control) were placed in the bottom the well. Non-adherent 24-well plates (#10156610, Fisher Scientific) were used. 300 μ L of platelet suspension were seeded in both sides of dHCM and CTR (glass surface) and incubated for 1 h at 37°C in a humidifier incubator with 5% CO₂. Then, samples were washed 3 times with PBS to remove non-adherent platelets. Samples were fixed with 10% formalin (for fluorescent imaging), 2.5 % glutaraldehyde (for SEM) and immersed in 2% Triton X-100 (for LDH quantification). Blood from three different donors was used in three independent assays.

VI-2.6. Measurement of platelet adhesion using lactate dehydrogenase (LDH) assay

Platelet adhesion was quantified by measuring the LDH released when adherent platelets were lysed with 2% (v/v) Triton X-100 (#A16046, Thermo Fisher Scientific) in PBS using the CyQUANT™ LDH Cytotoxicity Assay (#C20301, Invitrogen) according to manufacturers' instructions. A calibration curve was constructed using different dilutions of the seeded platelets, and the percentage of platelet adhesion on the different materials was determined from this calibration curve.

VI-2.7. Hemolytic activity assay

Blood was collected and centrifuged as described in subsection 2.5. PRP was used for platelet isolation, buffy coat was discarded, and the red blood cells (RBC) were washed three times with PBS. After each wash a centrifugation of 5 min at 3700 rpm was performed. RBC concentrate was diluted to a final concentration of 5 vol. %. 300 μ L of RBC suspension were seeded in both sides of dHCM (mounted in in cell crown inserts (#Z742380-12EA, Sigma)). PBS (0% lysis) and 1% SDS (82% lysis) were added to the RBC suspension and were used as the negative and positive control, respectively. After a 1 h incubation at 37°C in a shaking incubator chamber, samples were washed with PBS (3x) and fixed with 2.5% glutaraldehyde or centrifuged at 120 *g* for 10 min. 50 μ L of the obtained supernatants were transferred into a 96-well plate and the absorbance was measured at 540 nm. Readings were performed in duplicate. Blood from three different donors was used in three independent assays. To determine the hemolytic ratio the follow equation was used:

$$\text{Hemolytic ratio} = (\text{Sample} - \text{Negative control}) / (\text{Positive control} - \text{Negative control})$$

Hemolysis ratio was interpreted as follow: < 2% non-hemolytic, 2-5% slightly hemolytic, and > 5% hemolytic samples.

VI-2.8. Protein adsorption

For protein adsorption, platelet rich plasma (PRP) was provided by Centro Hospitalar de São João (Porto, Portugal) under an approved institutional board protocol (hospital ethical commission approval number 363/18). PRP was centrifuged at 800 *g* for 20 min at RT in order to obtain platelet poor plasma (PPP). 300 μ L of PPP or PBS (negative control) were added to both sides of dHCM (mounted in in cell crown inserts (#Z742380-12EA, Sigma)) and glass surfaces. After a 1 h incubation at 37°C, samples were washed three times with PBS. Then, samples were immersed in 1% SDS under agitation for 1 h and sonicated for 20 min. For protein quantification Micro BCA™ Assay Kit (#23235, Thermo Scientific) was used according with manufacturer's instructions. PPP was used as a positive control. Three independent donors were used in three independent assays.

VI-2.9. Microbiology

Microbiology tests were performed as previously described [28]. Both sides of dHCM membranes with 2.5 x 2.5 cm² were tested. Polypropylene films were used as positive controls (CTR (polypropylene films)). Briefly, *Staphylococcus aureus* (*S. aureus*; #25923, ATCC) were grown in tryptic soy broth media (TSB; #610053, Frilabo) in a shaking incubator at 37 °C overnight. Prior to inoculation, bacterial strains were sub-cultured in fresh TBS at 1:50 dilution and incubated for approximately 2 h in a shaking incubator (80 rpm) at 37 °C. Bacterial inoculum of 1 x 10⁴ colony forming units (CFU)/mL were used to completely submerged the membranes in the dish for 1 h at RT. Then, the bacterial suspension was removed, and the dish was washed three times with sterile PBS, for 10 s with an 80 rpm rotation in order to remove non-attached bacteria. Surfaces were dried for 1 h and contact plates were used to identify viable bacteria. In summary, contact plates were pressed onto inoculated membranes for 20 s and incubated overnight at 37 °C. Then contact plates were photographed and colonies were counted. The resulting colonies were log transformed and presented as log CFU/ contact plate (2.5 x 2.5 cm²).

VI-2.10. dHCM rolling

dHCM was rolled around a cylindrical mold in order to obtain a tube with two layers of dHCM. A surgical glue (Vetbond^{TM/MC}) was used to stick the dHCM. A dHCM conduit with 4 mm diameter was obtained.

VI-2.11. dHCM tube resistance to peristaltic movements

To verify if the dHCM tube was resistant to peristaltic movements, the 3D CultureProTM Bioreactor (#731754-0010, Electroforce) was used coupled to a peristaltic pump (#MS-2/6, Ismatec). The flow rate applied was 42.7 mL/min for 5 days using PBS with food coloring.

VI-2.12. Mechanical properties

Mechanical properties were assed using universal mechanical testing equipment (#5543, INSTRON) equipped with a 1kN load cell. At least five samples of tubular dHCM were used per assay. Longitudinal and circumferential tensile mechanical tests were performed. For the longitudinal tests, samples in tube form with a length of 2 cm and a diameter of 4 mm were used. For the circumferential test, tubes with 1 cm diameter were cut in 4 mm pieces. The stain rate was defined at 5 mm/min and a 10mm gauge length was used. Tests were finished when the specimens were ruptured.

VI-2.13. Scanning electron microscopy (SEM)

After sample fixation with 2.5% glutaraldehyde (#G5882, Sigma-Aldrich) in PBS, samples were washed three times with PBS. Then, they were dehydrated with increasing concentrations of ethanol (10% – 100%) and treated with HMD. Samples were air dried and mounted in SEM pins using carbon tape. The samples were coated with gold using a Sputter Coater (#EM ACE600, Leica). Micrographs were collected with a high-resolution field emission scanning electron microscope with focused ion beam (AURIGA COMPACT, Zeiss). Membrane's thickness was measured using the coupled software in 5 different samples.

VI-2.14. Fluorescence imaging and Immunocytochemistry

Phalloidin staining was performed in fresh tissue, fixed with 10% formalin. Samples were incubated with 1:200 Phalloidin-TRITC, (#P1951, Sigma-Aldrich), at RT for 1 h. For the von Willebrand factor staining (vWf) staining, samples were incubated with mouse anti-Von Willebrand Factor antibody (#ab201336, abcam) at 4 µg/mL, overnight at 4 °C in a humidified atmosphere. The goat anti-mouse IgG1 cross adsorbed secondary antibody, alexa fluor 488 (#A-21121, alfa gene) was used at 1:400 for 1 h at RT. Then, samples were analyzed under a fluorescence microscope with a coupled camera (Axio Imager Z1m, Zeiss). Images were further analyzed with the Zeiss Zen microscope software.

VI-2.15. Statistical analysis

Statistical analysis was performed using Graph Pad Prism 7. Shapiro-Wilk test was used to assess the data normality. As data followed a normal distribution, a parametric test was used, namely one-way or two-way ANOVA test followed by Turkey's multiple comparisons test. A $p < 0.05$ was considered statistically significant in the analysis of the results.

VI-3. RESULTS

VI-3.1. dHCM biocompatibility and recruitment of endothelial cells *ex ovo*

To assess the ability of dHCM to promote the formation of capillary-like structures *in vitro*, HUVECs were seeded in both sides of the membrane (reticular and trophoblast layer sides). The bottom of the well coated with Matrigel was used as positive control (CTR (Matrigel)). After 4 h, capillary-like precursors were not observed in any condition (Figure VI-1A). However, after 8 h capillary-like precursors were visible in positive CTR (Matrigel) condition and in dHCM reticular layer side, but they were inexistent in dHCM trophoblast layer side (Figure VI-1A, white triangles). At 12 h, it was possible to observe capillary-like precursors in dHCM trophoblast layer side. Nevertheless, in lower number that the ones observed in dHCM reticular side and CTR (Matrigel) (Figure VI-1A, white triangles). Angiopoietin 2 (ANG 2) was quantified at the same time-points (Figure VI-1B). The production of ANG 2 by HUVECs showed a tendency to increase overtime in all conditions. However, statistically significant differences were only observed in CTR (Matrigel) condition between 4 and 8 h ($p = 0.0024$) and 4 and 12 h ($p = 0.0003$) and in dHCM

reticular layer side between 4 and 12 h ($p = 0.0223$). Moreover, no statistically significant differences were observed between different conditions at the same time-point (Figure VI-1B). Four hours after seeding, dHCM reticular layer side presented the higher ANG2 production (116.49 ± 37.17 pg/mL) when compared with CTR (Matrigel) (80.66 ± 24.75 pg/mL) and dHCM trophoblast layer side (95.03 ± 4.27 pg/mL). This trend was altered at 8 h after seeding, where the higher ANG2 levels were associated with the CTR (Matrigel) condition (213.78 ± 43.46 pg/mL) followed by the dHCM reticular layer side (170.66 ± 68.69 pg/mL) and the dHCM trophoblast layer side (165.03 ± 13.33 pg/mL). The tendency was maintained at 12 h, CTR (Matrigel) (251.49 ± 52.24 pg/mL), dHCM reticular layer side (210.24 ± 10.48 pg/mL), and dHCM trophoblast layer side (168.16 ± 38.89 pg/mL) (Figure VI-1B).

A reendothelization assay was also performed in both sides of the dHCM and culture tissue coverslips were used as the assay control (CTR coverslip) (Figure VI-1C). After 48 h in culture, it was possible to observe a monolayer of endothelial cells (HUVECs) producing Von Willebrand Factor (VWF), a specific marker of endothelial cells (Figure VI-1C). This production was more pronounced in the dHCM reticular layer side, suggesting a faster reendothelization in this condition.

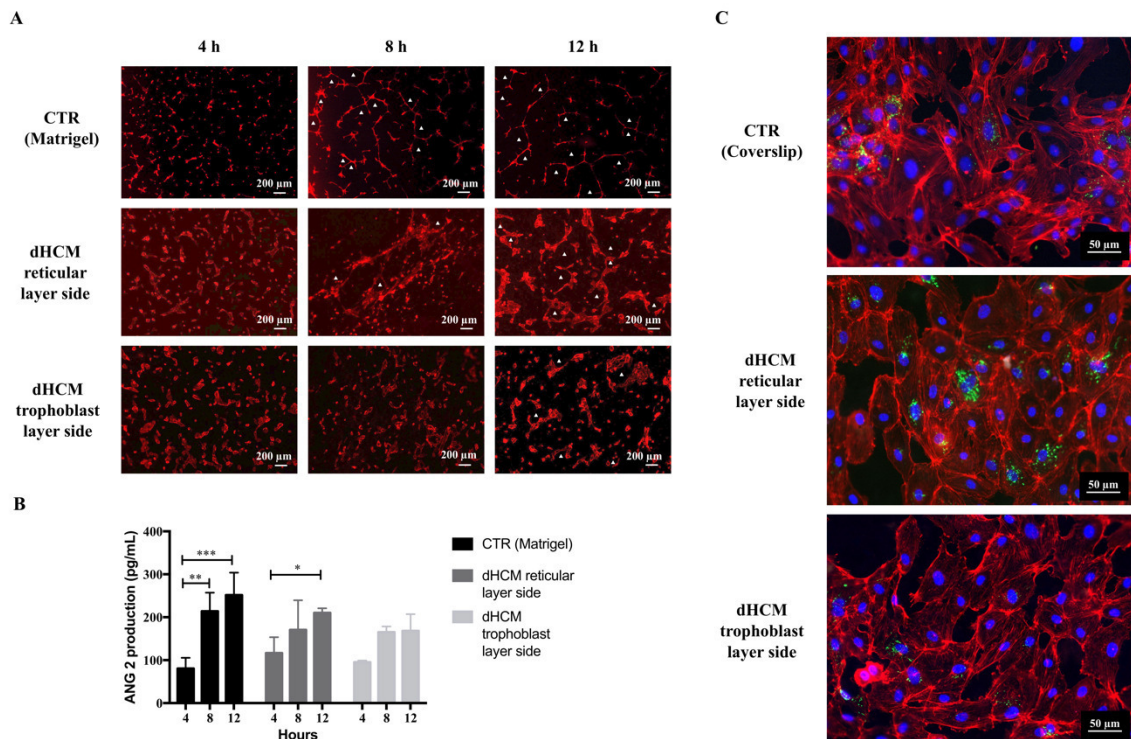


Figure VI-1 Endothelial cell compatibility. Phalloidin staining was used to assess the ability of HUVECs to form capillary-like structures (marked with a white triangle) in CTR (Matrigel) condition, dHCM reticular layer side and dHCM trophoblast layer side at 4, 8 and 12 h (A). For the same conditions at the

same time-points, angiopoietin 2 (ANG 2) production was quantified **(B)**. A reendothelization assay was also performed in three different conditions (CTR (coverslip), dHCM reticular layer side, and dHCM trophoblast layer side). After 48 h HUVECs were stained with phalloidin (red) and for Von Willebrand Factor (green) Where, * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

To study the ability of dHCM in inducing blood vessel formation *ex ovo*, a CAM assay was performed. Representative photographs of the chorionic and allantoic epithelium of CAM after 7 days of implantation, showing some dHCM retraction (Figure VI-2A). The control (CTR) condition (without material) represents the normal physiological behavior. No statistically significant differences were observed regarding the number of blood vessels formed, vascular density and vascular length density between the physiological condition (CTR (without material)) and both sides of dHCM (Figure VI-2B-D). Nevertheless, for all parameters, CTR (without material) condition was associated with higher values, followed by dHCM trophoblast layer side and dHCM reticular layer side.

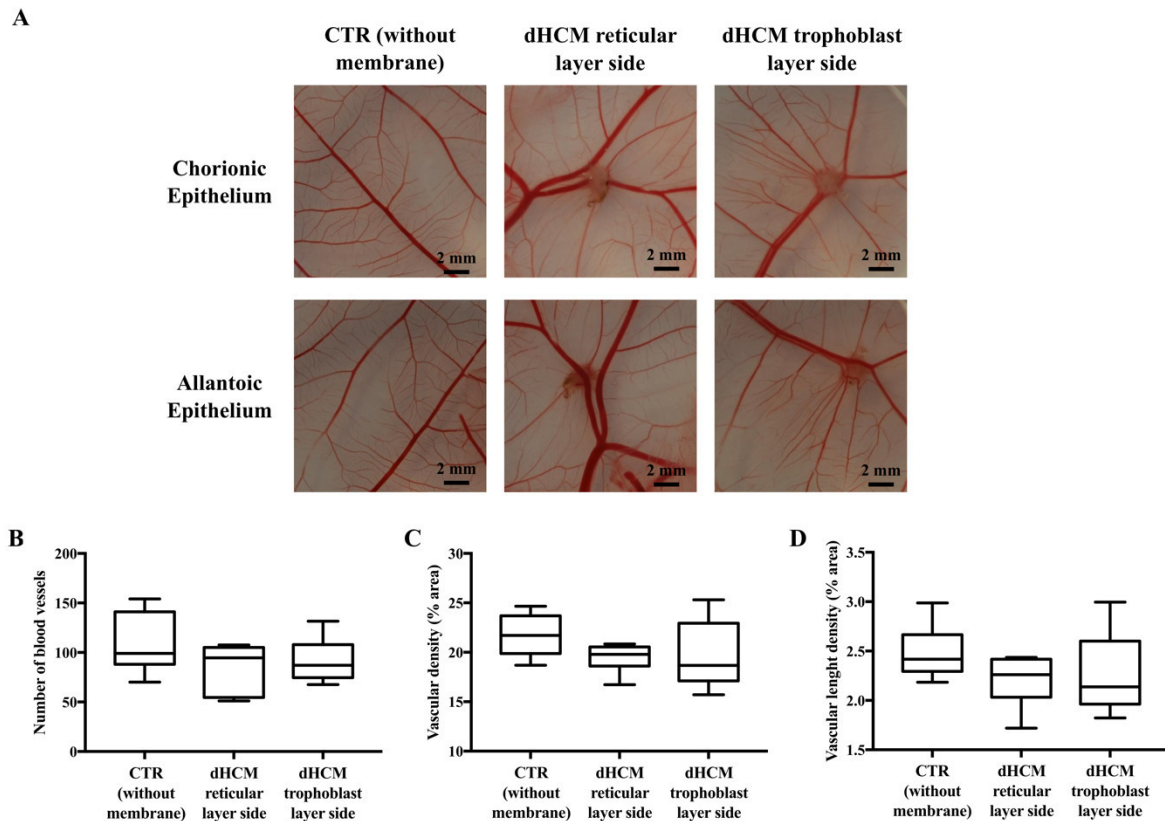


Figure VI-2 CAM Assay. Representative photographs of the chorionic and allantoic epithelium of *ex ovo* CAM after 7 days from implantation in control (CTR) condition (without material) and in both dHCM sides: reticular layer and trophoblast layer **(A)**. Several parameters were quantified in all conditions such as number of blood vessels **(B)**, vascular density **(C)**, and vascular length density **(D)**.

VI-3.2. dHCM thrombotic potential

To study dHCM thrombotic potential, plasma protein adsorption, platelet adhesion and aggregation analysis were performed (Figure VI-3). Glass surfaces were used as controls (CTR (glass surface)).

The higher plasma protein adsorption was observed in dHCM trophoblast layer side ($138.70 \pm 33.14 \mu\text{g}/\text{cm}^2$, data not shown), followed by dHCM reticular layer side ($97.31 \pm 43.17 \mu\text{g}/\text{cm}^2$, data not shown) and CTR (glass surface) condition ($34.18 \pm 53.09 \mu\text{g}/\text{cm}^2$, data not shown). These values were represented in percentage in relation to the initial protein amount (Figure VI-3A) and statistically significant differences were only observed between CTR (glass surface) condition and dHCM trophoblast side ($p = 0.00127$).

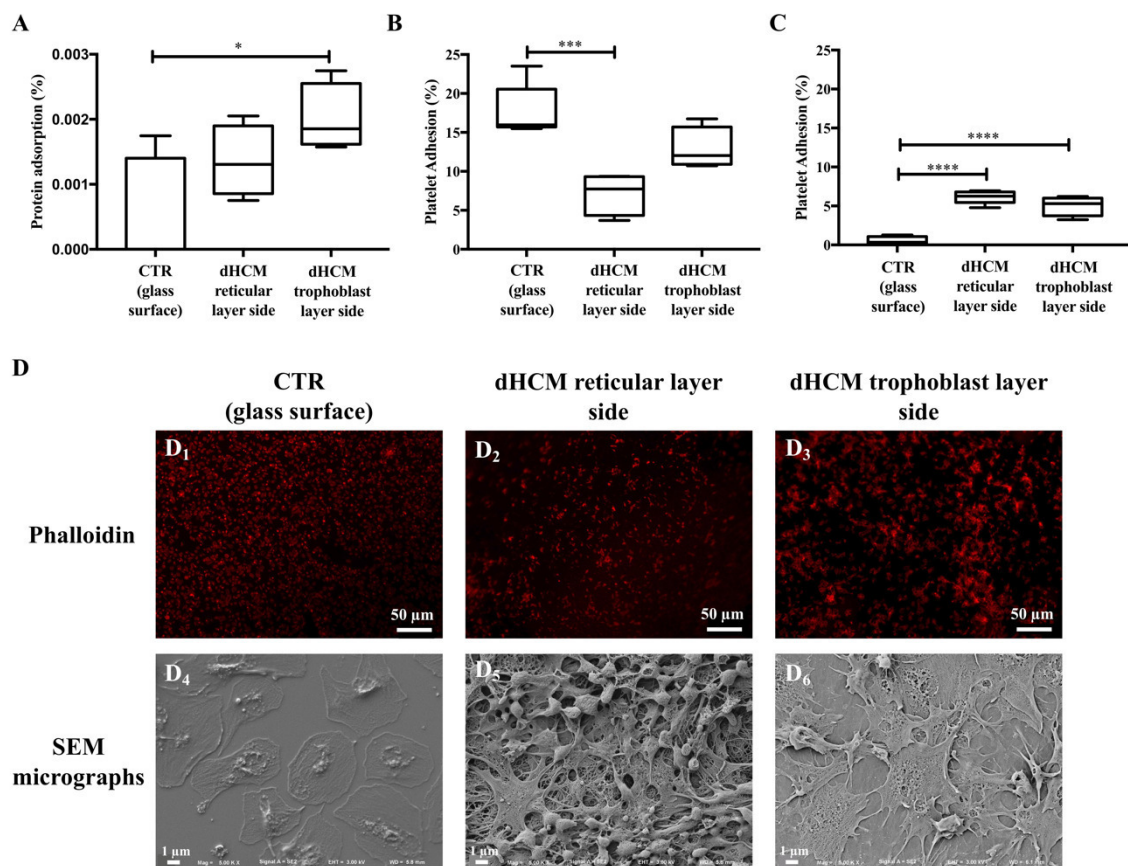


Figure VI-3 dHCM thrombotic potential. Plasma protein adsorption was quantified in CTR condition (glass surface) and in both sides of dHCM (**A**). Platelet adhesion was analyzed for the same conditions using isolated platelets (**B**) and platelet poor plasma (PPP) (**C**). Isolated platelets on the three different surfaces were stained with phalloidin (**D₁-D₃**). Moreover, SEM micrographs were obtained to verify platelet morphology (**D₄-D₆**). Where, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Platelet adhesion and aggregation studies were performed with isolated platelets (Figure VI-3B) and platelet rich plasma (PRP) (Figure VI-3C). Interestingly, when isolated, platelet adhesion was higher in CTR (glass surface) condition when compared with dHCM reticular layer side ($p = 0.0009$) and dHCM trophoblast side ($p = 0.849$) (Figure VI-3B). An opposite trend was observed when PRP was used, showing a smaller platelet adhesion in CTR (glass surface) when compared with dHCM reticular layer side ($p < 0.0001$) and dHCM trophoblast layer side ($p < 0.0001$) (Figure VI-3C). A decrease in platelet adhesion was observed when PRP was used in CTR (glass surface) ($0.55 \pm 0.49\%$ (PRP) vs. $17.69 \pm 3.35\%$ (isolated platelets)) and dHCM trophoblast layer side ($5.02 \pm 1.26\%$ (PRP) vs. $12.88 \pm 2.68\%$ (isolated platelets)). Regarding dHCM reticular layer side, platelet adhesion was similar when using PRP ($6.11 \pm 0.83\%$) or isolated platelets ($7.14 \pm 2.69\%$) (Figure VI-3B-C).

Altogether, these results suggested that dHCM presents anti-thrombogenic activity, mainly in the dHCM reticular layer side. To further verify this tendency, isolated platelets were stained with phalloidin and analyzed by SEM (Figure VI-3D). Phalloidin staining (Figure VI-3 D₁-D₃) corroborated the previous results regarding platelet adhesion (Figure VI-3A). A higher platelet density was observed in CTR (glass surface) condition (Figure VI-3D₁) followed by dHCM trophoblast layer side (Figure VI-3D₃) and dHCM reticular layer side (Figure VI-3D₂). By SEM micrographs it was possible to analyze the platelets activation. In their resting state (non-thrombotic), platelets have a smooth discoid shape. However, platelet activation triggers morphological changes characterized by 4 stages: dendritic, spread-dendritic, spread, and fully spread [29]. On the CTR (glass surface), platelets demonstrated a fully spread morphology, indicating platelet activation (Figure VI-3D₄). On dHCM trophoblast layer side, platelets with a dendritic and a spread dendritic morphology were observed, also indicating platelet activation and thrombotic activity (Figure VI-3D₅). Although, in dHCM reticular layer side, platelets with a spread dendritic morphology were identified, discoid shape platelets were also observed and in a higher number, suggesting a non-thrombotic activity for dHCM reticular layer side (Figure VI-3D₅).

VI-3.3. dHCM hemolytic potential

To study the dHCM hemolytic potential, red blood cells (RBC) were seeded in the membrane. Glass surfaces were used as control (CTR (glass surface)) (Figure VI-4). The hemolytic ratio was $0.59 \pm 0.13\%$ for CTR (glass surface), $0.42 \pm 0.12\%$ for dHCM reticular layer side, and $0.16 \pm 0.11\%$ for dHCM trophoblast layer side (Figure VI-4A). So, all surfaces were non-hemolytic ($< 2\%$). As expected, statistically

significant differences were observed between assay positive CTR (1% SDS, hemolytic ratio = 100%) and assay negative CTR (PBS) ($p < 0.0001$), CTR (glass surface) ($p < 0.0001$), dHCM reticular layer side ($p < 0.0001$), and dHCM trophoblast side ($p < 0.0001$) (Figure VI-4A). Furthermore, by SEM micrographs it was possible to identify normal RBC (round cells with an indented center and biconcave disc shape) and echinocytes (altered RBC covered by small, evenly spaced thorny projections) [30] (Figure VI-4B). Altogether, these results demonstrated the hemocompatibility of both sides of dHCM.

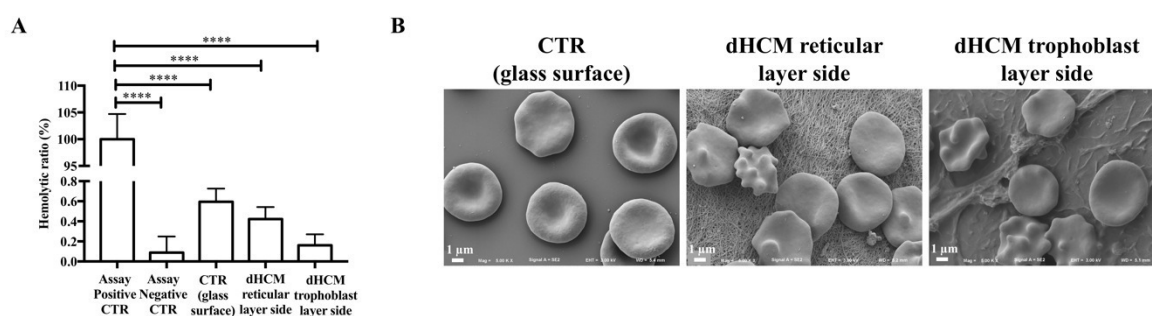


Figure VI-4 dHCM hemolytic potential. The hemolytic ratio was obtained for the CTR (glass surface) condition and for both sides of dHCM. For the assay positive and negative control, 1% SDS and PBS were added to the RBC suspension, respectively **(A)**. SEM micrographs of RBC seeded into the different surfaces were obtained **(B)**. Where **** $p \leq 0.0001$

VI-3.4. dHCM antibacterial properties

The activity of both dHCM sides against *S. aureus* adherence and attachment was assessed by an immersion assay. Polypropylene films were used as positive controls (CTR (polypropylene films)) (Figure VI-5). It was possible to verify a reduction in CFU in dHCM (Figure VI-5A) when compared to CTR (polypropylene films) ($p = 0.0017$ vs. dHCM reticular layer side, $p = 0.0003$ vs. dHCM trophoblast layer side) (Figure VI-5B). No differences were observed between both sides of dHCM ($p = 0.7020$) (Figure VI-5B).

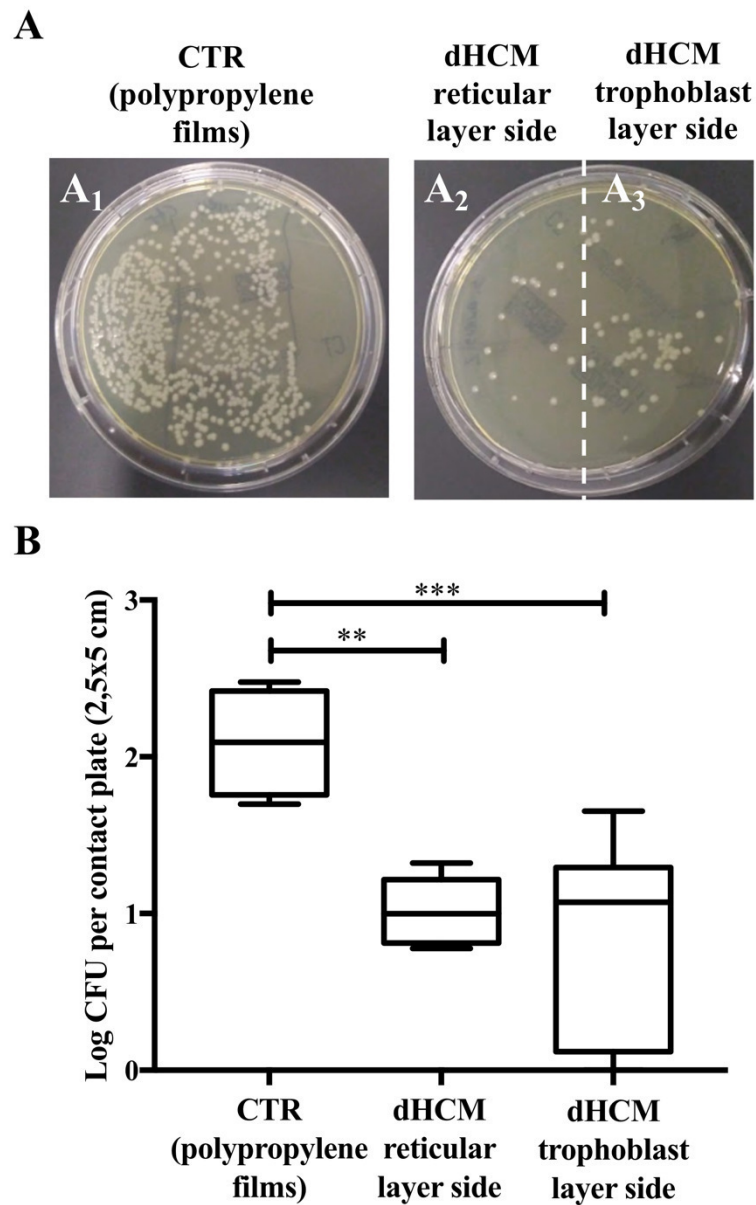


Figure VI-5 dHCM resistance to infection. dHCM antibacterial activity against *S. aureus* adherence and attachment, photographs of contact plates in CTR (polypropylene films) (**A₁**), dHCM reticular layer side (**A₂**) and dHCM trophoblast layer side (**A₃**). CFU quantification in all surfaces (**B**). Where, ** $p \leq 0.01$ and *** $p \leq 0.001$.

VI-3.5. Tubular construct and mechanical properties

To use the dHCM as a vascular graft, it is important to obtain a tubular shape scaffold resistant to peristaltic movements and flow rate. So, the dHCM was rolled in order to obtain a tube with 3-4 mm diameter (Figure VI-6A). The length of the tube may vary depending on the need (Figure VI-6B). Nevertheless, it will be limited to the size of the chorion membrane removed from the human placenta. In this work, due to the mold used, tubular constructs with 6 cm length were obtained. The wall thickness of the tubular constructs was also determined, being $61.68 \pm 9.01 \mu\text{m}$ (Figure VI-6C). As vetbond was used to glue to the dHCM together into a tubular structure, SEM micrographs were obtained to verify the existence of delamination of the graft layers. In the region where the graft started to be rolled, there was delamination of the graft layers (Figure VI-6D). SEM micrographs of the inner and outer layer of the vascular construct were obtained (Figure VI-6E and 6F, respectively), showing the different topographies that characterize the two dHCM sides (reticular and trophoblast layer side).

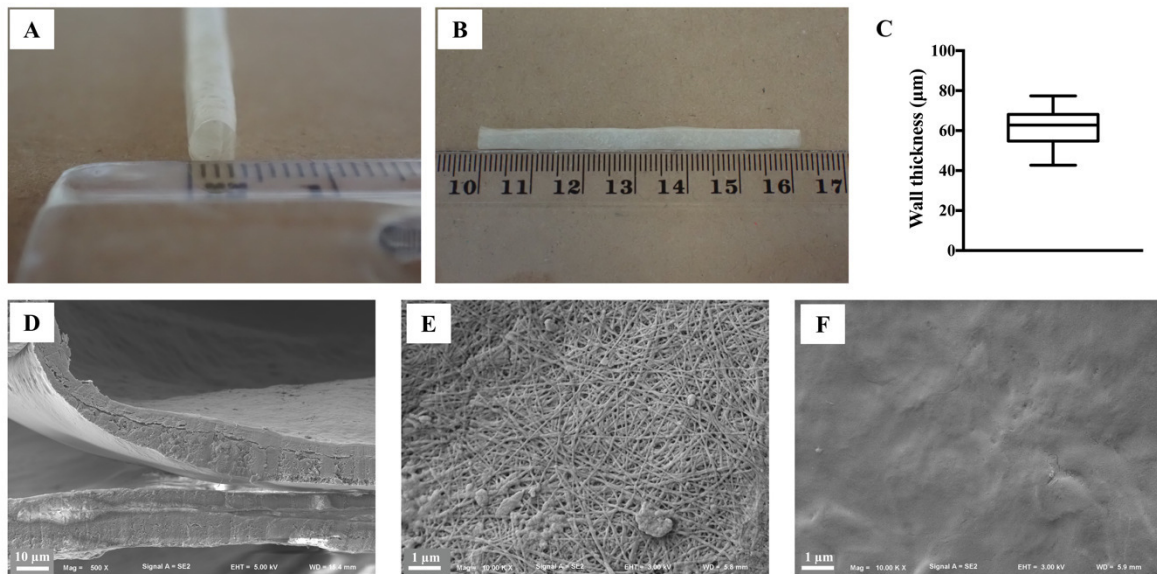


Figure VI-6 dHCM tubular structure. dHCM was rolled creating a tube with 3-4 mm diameter **(A)** and variable length **(B)**. The walls of the dHCM tube were characterized regarding its thickness **(C)**. Representative SEM micrographs of a transversal cut of the dHCM tube **(D)**, of the inner side of the dHCM tube, the reticular layer side **(E)**, and of the outer side the dHCM tube, the dHCM trophoblast layer side **(F)**.

The dHCM in a tubular form was mounted in the 3D CulturePro™ Bioreactor and a flow rate of 42.7 mL/min was applied using a peristaltic pump (Figure VI-7A). After 5 days, the dHCM tube was not

showing evidence of loss of structural integrity nor leaking the fluid (Figure VI-7B), suggesting its resistance to peristaltic movements. Moreover, the longitudinal and circumferential Young's modulus and ultimate tensile strength of dHCM constructs were determined (Figure VI-7C and 7D). The longitudinal Young's modulus was 128.70 ± 22.72 MPa and the circumferential Young's modulus was lower, 40.84 ± 11.51 MPa (Figure VI-7C). For the ultimate tensile strength, the same tendency was observed, being 13.42 ± 3.26 MPa for the longitudinal measures and 3.47 ± 0.71 MPa for the circumferential measures (Figure VI-7D). In figures 7E to 7H, it is possible to observe that while bending the tubular graft, the flow is not blocked, suggesting that the vascular graft do not kink, despite bending.

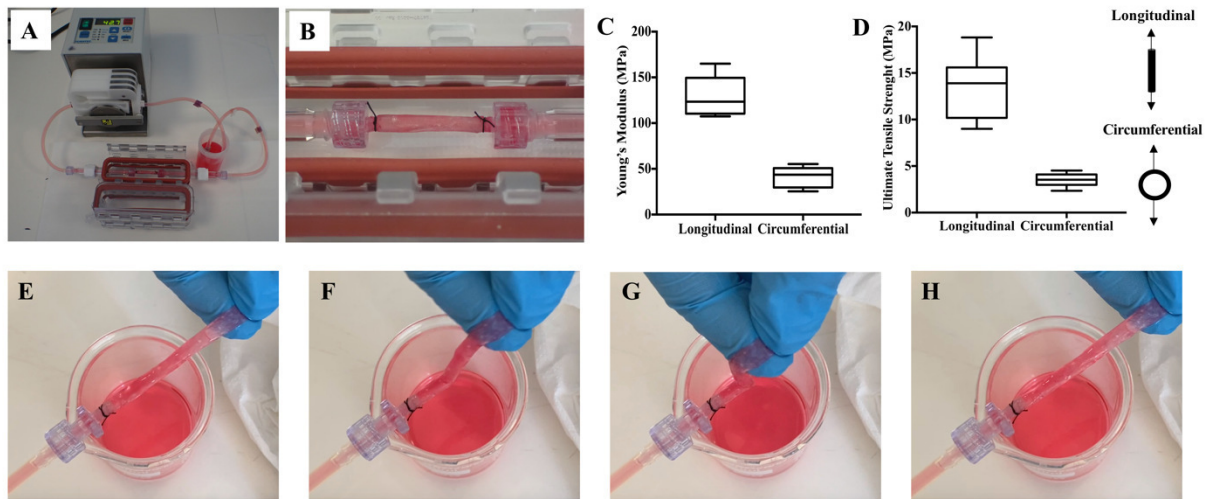


Figure VI-7 Mechanical characterization of the dHCM tube. dHCM tube was mounted in 3D CulturePro™ Bioreactor attached to a peristaltic pump and submitted to a flow rate of 42.7 mL/min (**A-B**). Longitudinal and circumferential Young's modulus (**C**) and ultimate tensile strength (**D**) of dHCM were determined. Despite bending the tubular graft, the flow is not blocked (**E-H**).

VI-4. DISCUSSION

A vascular graft should be compatible with endothelial cells and should be able to promote endothelization of its inner surface. Moreover, it should present blood compatibility, resistance to infection and appropriate mechanical properties [6,25,26]. In this work it was demonstrated that dHCM presents all these characteristics, mainly in the dHCM reticular layer side.

dHCM compatibility with endothelial cells was shown in accordance with previous works [22]. Moreover, the reticular layer side of the dHCM was able to induce the formation of capillary-like precursors *in vitro*, showing not only HUVECs survival but also the maintenance of its functionality [31] when seeded in dHCM reticular layer side. In the positive CTR (Matrigel) condition at 8 and 12 h, HUVEC's capillary-like precursors were clearer when compared with both sides of dHCM. This may be related with the surface and its influence in imaging but also with the use of Matrigel as a control, representing a tumor-like environment and consequently a richer substrate [32]. The production of ANG2 by endothelial cells is associated with tyrosine-protein kinase (Tie2) activation that is critical for endothelial cell functions, such as the induction of tube formation [33]. It was shown an increase in endogenous ANG2 overtime, suggesting the maintenance of endothelial cells function in culture. Moreover, after 48 h of culture, a monolayer of endothelial was observed, with a higher production of VWF in the dHCM reticular layer side. VWF staining was observed as intracellular green dots localized around the cell nuclei, that is characteristic of healthy endothelial cells [34]. These results suggest that when seeded on dHCM reticular layer side, HUVECs maintained their endothelial cell phenotype specificity [34]. Furthermore, this rapid reendothelization is very important to create an endothelial cell lining in the vascular graft that will not only function as a protective and thromboresistant barrier, but it will also control vessel tone, platelet activation and leucocyte adhesion [6].

After implantation, a vascular graft should recruit endothelial cells *in vivo* in order to be repopulated and mimic an autologous blood vessel while the graft degrades [25,26]. CAM assay represents a rapid, simple, and cost-effective *ex ovo* test to screen the angiogenic potential of biomaterials [35,36]. By the CAM assay, we were able to demonstrate the ability of dHCM to recruit functional endothelial cells *ex ovo*. The CTR (without membrane) represented the physiological condition. Although, dHCM reticular layer side was associated with a slightly lower number of blood vessels, vascular density and vascular length density, no statistically significant differences were observed between the three conditions. These results suggested that both sides of dHCM had a non-toxic effect over endothelial cell recruitment.

Vascular grafts interact directly with blood. So, its blood compatibility is imperative to avoid platelet adhesion and activation, coagulation, and thrombosis [37]. After the interaction blood-material, the first event occurring on biomaterial surfaces is a rapid plasma protein adsorption that mediates the subsequent biological responses [37–40]. We demonstrated that both sides of dHCM were associated with a low protein adsorption, as well as, with a rapid endothelial cell recruitment and reendothelization

rate, suggesting a lower probability of thrombus formation and inflammation [40]. Platelet adhesion and aggregation function as a pro-thrombotic marker since platelet deposition may difficult endothelization and consequently facilitate the development of thrombotic events [41]. Platelets tend to adhere more frequently to flat surfaces like glass [42], this justify our results regarding isolated platelets, where platelet adhesion is higher in CTR (glass surface) surfaces when compared with both sides of dHCM. Moreover, by using PRP, it was possible to verify the influence of plasma proteins adsorption in platelet adhesion. In general, when using PRP, platelet adhesion was lower, demonstrating the anti-thrombotic activity of dHCM. These results were corroborated by the analysis of the platelets morphology on the surfaces. dHCM reticular layer side was the only surface (when compared with CTR (glass surface) and dHCM trophoblast layer side) that presented discoid shape platelets, suggesting a non-thrombotic activity for dHCM reticular layer side. On the other hand, material-blood interaction may be associated with hemolysis. Hemolysis is characterized by the loss of RBC membrane integrity, leading to hemoglobin leakage into blood plasma that can trigger specific pathophysiologic events [43]. Our results demonstrated the hemocompatibility of dHCM since the hemolytic ratio was inferior to 2%. Corroborating these results, SEM micrographs demonstrated normal RBC on the surfaces of both sides of the dHCM. The presence of echinocytes may be explained by the exposure to anticoagulants when blood was collected [44].

Although uncommon, vascular graft infection is a serious complication associated with significant morbidity and mortality. All microorganisms such as gram-positive, gram-negative, anaerobic, and fungal, have the potential to infect vascular grafts [45]. However, *S. aureus* is the most prevalent microorganism associated with vascular graft infection [45,46]. In this work, we demonstrate that both dHCM sides were associated with a low adherence and attachment of *S. aureus*.

The replacement of large diameter vessels (> 6 mm) has been successfully assured by synthetic polymers [47]. However, most of the vessels in the peripheral, cerebral, and cardiac vasculature have diameters of less of 6 mm (small diameter vessels). The replacement of these vessels presents several challenges, since the use of synthetic grafts is associated with rapid thrombus formation, intimal hyperplasia, continued risk of bacterial colonization, and in pediatric patients, the synthetic grafts are not able to grow and adapt [47]. In this work we were able to create a tubular structure using dHCM with 3-4 mm in diameter. However, by using different molds, other diameters may be achieved. Taking the previous results in consideration, we provided evidence that the dHCM reticular layer side is the most appropriate to be in the inner side of the tubular graft. The dHCM was rolled using a concentric rolling

technique previously described for decellularized human amniotic membrane [19]. There are different rolling technologies, combining textiles techniques with biological materials [48]. Nevertheless, since in this work, dHCM sheets were used, the concentric rolling technique was considered more adequate to produce the tubular constructs. A surgical glue, Vetbond, was used to attach the dHCM sheets. Vetbond is a tissue adhesive made of cyanoacrylates used in veterinary applications. Although non-toxic, Vetbond is not approved for use on humans. So, in future works, other surgical glues approved for use on humans, should be tested, such as Dermabond (made of different cyanoacrylates and approved for use on humans) or a different type of tissue adhesive, such as fibrin glues.

A vascular graft should have a long-term durability and should be resistant to fatigue [49]. In this work, a bioreactor was used to demonstrate dHCM resistance to flow rate and peristaltic movements. A flow rate of 42.7 mL/min was used, similar to the ones reported for internal mammary artery [50]. Results demonstrated that despite there is a gap between the inner and the outer layer of the dHCM vascular graft in the region where it started to be rolled, no turbulence or leaking was observed when the fluid was added. Longitudinal and circumferential mechanical properties of the dHCM vascular graft were determined. The longitudinal ultimate tensile strength was 13.42 ± 3.26 MPa with a Young's modulus of 128.70 ± 22.72 MPa. For the circumferential samples these values were lower, 3.47 ± 0.71 MPa of ultimate tensile strength and 40.84 ± 11.51 MPa of Young's modulus. These values are similar to the ones reported for the saphenous vein (longitudinal: 13 MPa ultimate tensile strength and 130 MPa Young's modulus; circumferential: 3 MPa ultimate tensile strength and 43 MPa Young's modulus) [51], the gold standard autologous graft for small caliber vessel replacement [5,52]. Finally, it was possible to demonstrate that the dHCM vascular graft do not kink, despite bending, avoiding the blocking of the blood stream and consequently the cause of thrombotic events. Further studies regarding wall shear stress, turbulence, transmural pressure, pulsatile pressure, and wall motion [53] should be performed in order to fully characterize the performance of the dHCM vascular graft.

Hybrid vascular grafts have been proposed aiming to combine the ECM ability to provide cellular cues and synthetic materials' mechanical properties [54,55]. Nevertheless, due to the production techniques applied, they are associated with higher costs and longer processing times, when compared with dHCM.

Owing to the body's natural healing process, when implanted *in vivo*, the vascular graft will be remodeled *in situ*. Further *in vivo* studies would be important to verify if the degradation rate of dHCM *in vivo* will match the rate of vascular tissue regeneration in order to avoid aneurysms or vascular rupture [1,6].

VI-5. CONCLUSIONS

This work demonstrates that dHCM has the needed characteristics to be used in the production of vascular grafts. Both sides of dHCM shown to be compatible with endothelial cells (HUVECs) *in vitro* and *ex ovo*, to present blood compatibility and to have antibacterial properties against *S. aureus* adherence and adhesion. Nevertheless, dHCM reticular layer side was associated with a faster reendothelization, a lower platelet adhesion and activation, and with a better hemocompatibility. So, a dHCM tube with 3-4 mm in diameter was fabricated with the reticular layer side facing the inner side of the tube. This vascular graft was characterized, and its mechanical properties were similar to the ones of the saphenous vein, the gold standard for autologous small diameter vessel replacement. Further studies regarding hemodynamic wall shear stress and strain should be performed in order to fully characterize the dHCM vascular graft. Moreover, it would be important to verify if the degradation rate of dHCM *in vivo* would match the rate of vascular tissue regeneration, avoiding aneurysms or vascular rupture.

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VI-7. ACKNOWLEDGEMENTS

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Chapter VII

Novel diaphragmatic hernia porcine model to study the influence of subject growth: a comparison study between Gore-Tex® and biodegradable membranes

Chapter VII

Novel diaphragmatic hernia porcine model to study the influence of subject growth: a comparison study between Gore-Tex and biodegradable membranes^s

ABSTRACT

A novel diaphragmatic hernia animal model was established using thoracoscopy in sexually immature farm pigs, allowing for characterization of the effect of subject growth in defect size. This model was used to compare the performance of two new membranes, (polycaprolactone electrospun fibrous mesh (PCLem) and decellularized human chorion membrane (dHCM)) against the clinical gold standard (Gore-Tex®) for diaphragmatic hernia repair. Patch repair for congenital diaphragmatic hernia (CDH) is associated with infection, adhesions, hernia recurrence and long-term musculoskeletal sequels. To overcome current limitations of using non-absorbable materials (such as Gore-Tex®), which are mainly associated with poor tissue regeneration, we developed two novel membranes based on the therapeutic potential of the extracellular matrix (ECM). One membrane is of synthetic origin (PCLem), although able to mimic the ECM fibrous morphology; and the other is a decellularized human chorion membrane (dHCM), presenting the architecture and some components of natural ECM. It was demonstrated that PCLem and dHCM were associated with better tissue integration, but also with a higher hernia recurrence (50% and 100%, respectively), when compared to Gore-Tex® (0% recurrence). Further long-term studies and improvements in animal model and membrane design and applications are needed to create a solution that promotes tissue regeneration *in situ* without hernia recurrence.

^s This chapter is based on the following publication:

Frazão, L. P.; Scuglia, M.; Miranda, A.; Martins, A.; Barbosa-Sequeira, J.; Coimbra, D.; Longatto-Filho, A. Reis, R.L.; Nogueira-Silva, C.; Correia-Pinto, J.; Neves, N.M. Novel diaphragmatic hernia porcine model to study the influence of subject growth: a comparison study between Gore-Tex® and biodegradable membranes(submitted).

VII-1. INTRODUCTION

Multiple surgical strategies for congenital diaphragmatic hernia (CDH) have been reported, relying heavily on the size of the diaphragmatic defect and thus susceptible to the surgeon's individual discretion. To standardize procedures, the Congenital Diaphragmatic Hernia Study Group (CDHSG) has developed a classification based on defect size [1]. CDHSG reports have also demonstrated a close association between defect size and morbidity and mortality [1,2]. In patients with large defects (large B and C defects) [1] or complete diaphragmatic agenesis (D), patch repair (PR) remains the only viable surgical option. According to CDHSG, PR accounts for 52.5% of CDH cases [3] and stands consistently as a predictor of adverse outcomes. In fact, PR is associated with infection, adhesions and hernia recurrence, the latest occurring in up to 50% patients up to the age of 3 months [4]. Long-term sequels are often reported, such as scoliosis (27%) and chest wall deformities (50%) [5], which result from the inability of the prosthetic materials to mimic diaphragmatic properties and expand accordingly in a setting of rapid child growth [6–8].

Thoracoscopic repair of CDH has been performed for the last two decades. When compared to open surgery, it has been associated with fewer postoperative ventilator days, less use of analgesics [9], shorter hospital stays, fewer intra-abdominal complications, better wound cosmesis lower incidence of subsequent scoliosis and chest deformities [10]. However, a higher recurrence rate after thoracoscopic repair was reported (0 - 24% *vs.* 0 - 11% in open repair) [11]. This was associated with surgery-related technical factors, such as the surgeon learning curve. In fact, a systematic review by Jancelewicz *et al* [12] noted a decrease in recurrence rates from 50% prior 2008 to 25% after 2008, suggesting that surgical skills were improving. On the other hand, when compared with open surgery, minimally invasive CDH repair has been associated with lower mortality rate. Nevertheless, these results have been related with selection bias, i.e., groups where minimally invasive repair was used, may have included less severe cases [10]. In 2017, Putnam *et al*/stratified CDH patients by risk and demonstrated that although minimal invasive repair (when compared with open surgery) was associated with shorter hospital stays and fewer adhesive small bowel obstruction, it was still associated with higher recurrence rates [13]. Thus, the benefits of minimal invasive surgery in CDH patients, such as laparoscopy and thoracoscopy, are still inconclusive. Thoracoscopic repair of CDH is not a routine treatment for every neonate and it is important to select suitable cases for the use of this technique [10,13].

A variety of prosthetic materials has been developed for PR [14]. Polytetrafluoroethylene (PTFE; Gore-Tex®), a synthetic non-absorbable polymer, is a durable and resistant material and therefore is considered the gold-standard for diaphragmatic replacement [15]. However, it is associated with poor tissue integration and high

recurrence rates associated with the subject growth [16–18]. Similarly, other synthetic materials, such as Seamdura® are also associated with high recurrence rates [19]. Several biological materials were applied in PR (both mixed or alone), such as small intestine submucosa (SIS, Surgisis®) [16–18,20], autologous biosheets [19], decellularized diaphragms [21,22], acellular collagen matrices [17,23–26], acellular dermal matrix (AlloDerm) [27] and porcine dermal collagen (Permacol®) [28]. Despite advantages such as low immunogenicity and tissue remodeling, absorbable biomaterials are still a limited choice for diaphragmatic replacement due to patch thinning and incomplete tissue ingrowth and consequently a higher-than-desired re-herniation rate. Blend materials with synthetic and biological components were also proposed for CDH [29–32]. Although studies show better tissue integration, recurrence rates are still sub-optimal.

Aligned on the therapeutic potential of extracellular matrix (ECM)-based membranes [33,34], two different patches were analyzed in this study: one synthetic in origin (mimicking the ECM morphology) and other biological (maintains the architecture and some ECM components). The synthetic patch is a fibrous membrane made of polycaprolactone (PCL), randomly organized in a mesh-like structure and fabricated using the electrospinning technique (PCLem) [35]. Based on previous studies with diverse cell types [36–38], it is hypothesized that PCLem should encourage host angiogenesis, cell proliferation, migration, and differentiation, eventually replacing the graft with constructive connective tissue. The biological membrane results from the decellularization of the human chorion membrane, as previously described [39]. The decellularized human chorion membrane (dHCM) is a compact and acellular membrane that was shown to be biocompatible both *in vitro* and *in vivo*, and to be integrated by host tissue [40].

Therefore, the aim of this study is to mimic the condition of neonatal PR in CDH by the proposal of a novel diaphragmatic hernia model using thoracoscopic techniques in sexually immature young farm pigs. Moreover, two novel membranes (PCLem and dHCM) will be compared to the clinical gold-standard (Gore-Tex®) for diaphragmatic hernia treatment.

VII-2. MATERIALS AND METHODS

This study was performed following the EU Directive 2010/63/EU and approved by the Animal Ethics Committee of the institution where the study was performed (ORBEA EM.ICVS-I3Bs_010/2019). All personnel involved in the procedures were approved as competent for animal experimentation by the DGAV (Portuguese general directorate of food and veterinary). Gore-Tex® was compared with polycaprolactone electrospun fibrous mesh (PCLem) and decellularized human chorion membrane

(dHCM) [39,40] in the repair of a surgically created diaphragmatic defect in a growing animal model, in accordance with Figure VII-1.

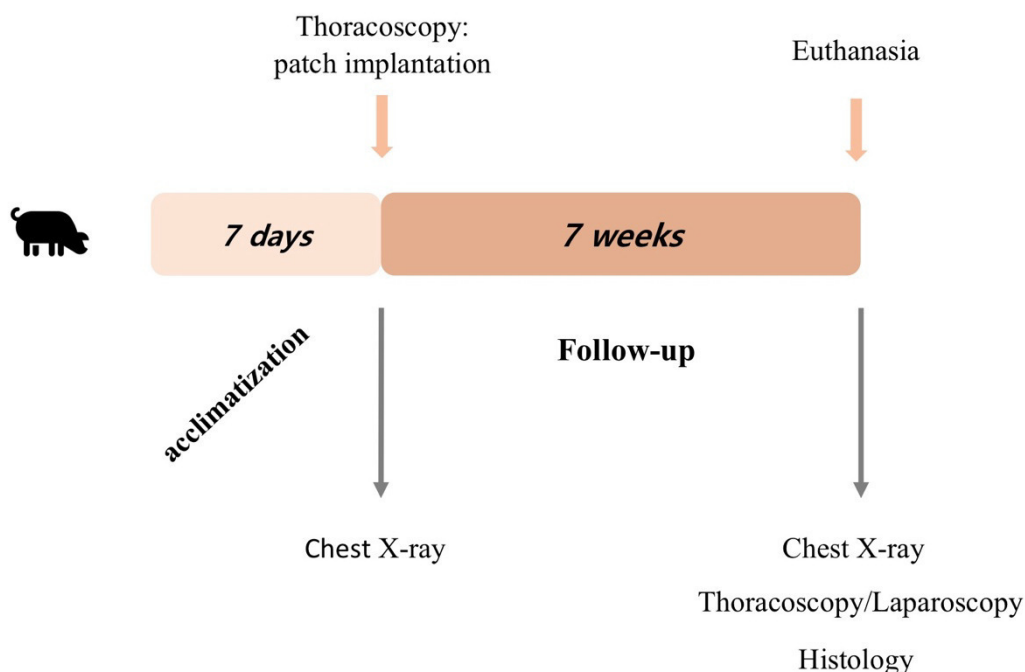


Figure VII-1 Experimental timeline. Farm male pigs were acclimatized for 7 days and then submitted to a chest X-ray and thoracoscopic surgery for patch implantation. Seven weeks post-surgery, animals were submitted to a second chest X-ray, thoracoscopy, laparoscopy and were euthanized.

VII-2.1. Patch preparation

Gore-Tex® Soft Tissue Patch was purchased from W. L. Gore & Associates (Delaware, USA). PCLem were prepared using a solution of 20% PCL with a solvent mixture of chloroform and dimethylformamide (8:2 ratio). Then, the PCL solution was electrospun by applying a voltage of 15.5 kV, a needle tip to ground collector distance of 20 cm, and a flow rate of 2 mL/h. After the complete processing of 1 mL of PCL solution (30 min), PCLem was allowed to air dry for 1 day. dHCM were obtained as previously described [39], following sample collection and its processing in accordance with the protocol approved by the ethical committees of Hospital de Braga and University of Minho (CESH 030/2016, SECVS 136/2015, respectively).

VII-2.2. Animal care and use

Fourteen farm male pigs (*Sus scrofa domesticus*) with approximately 6 weeks of age (6.54 ± 0.57 kg body weight) arrived at our facility 7 days prior to the study for acclimatization. Animals were housed in socially stable groups of 2 or 3 in each pen, and visual contact between pens was allowed. Dry pellet food was provided once a day; water was available *ad libitum* through automatic drinkers and bowls. Animals were housed in a facility with controlled temperature (24 ± 2 °C) and automatic air renewal. Wood shavings were provided for bedding and enrichment. Additional enrichment material was provided, including fresh fruit, ropes and balls. Animals were randomly assigned to 3 different experimental groups of 4 animals each, determined by the type of patch used during thoracoscopic diaphragmatic repair (Gore-Tex®, PCLem and dHCM). Two additional animals were used as age and weight-matched controls and submitted to thoracoscopy without creation of diaphragmatic defect (Sham group).

VII-2.3. Chest x-rays

Preoperatively and at the end of the protocol (euthanasia), all animals were submitted to a chest X-Ray evaluation under sedation in order to document lung pathology (i.e., atelectasis, infection), hernia recurrence or diaphragmatic eventration, and skeletal deformations such as scoliosis or thoracic wall deformities.

VII-2.4. Anesthesia and analgesia

All surgical procedures were performed under general anesthesia with endotracheal intubation and mechanical ventilation. An 8 h fasting period was performed, while water was provided until sedation time. Animals were anesthetized with a combination of ketamine (20 mg/kg, intramuscularly (IM)), xylazine (2 mg/kg), and atropine (0.04 mg/kg, IM), followed by propofol (4 mg/kg, intravenously (IV)) and maintained with continuous propofol infusion ($20 \text{ mg kg}^{-1} \text{ h}^{-1}$, IV) and buprenorphine (0.05 mg/kg). Additional intra and post-operative analgesia was provided by non-steroidal anti-inflammatory (NSAID) Meloxicam (0.4 mg/kg, IM). Maintenance of water and electrolyte balance was provided by intravenous administration of isotonic fluids ($10 \text{ ml kg}^{-1} \text{ h}^{-1}$, IV). After induction, an electronic microchip transponder was implanted at each animal for individual identification (*LifeChip*, *BioThermo*). The animals were

checked for identification by portable readers (*SURESense*, *SUREFlap*) at every experimental timepoint afterwards.

VII-2.5. Surgical technique

The order of surgeries for different patch-types was randomized as well as the animals subjected to the different treatment groups. All surgical interventions were performed under sterile conditions and continuous cardiorespiratory monitoring. Animals were positioned in right lateral decubitus, with the left foreleg retracted cranially. A nasogastric tube was placed. A 5 mm port was placed in the 6th intercostal space aligning with the shoulder joint, for introduction of a 5 mm 30° telescope. Two working ports were placed in the 7th intercostal space under endoscopic vision, one 3 mm port along a line passing through the tip of the left scapula and the other 5 mm port approximately 5 cm from the left costal arch, allowing access to the diaphragm (Figure VII-2A). Capnothorax was established with an inflation rate of 1 L/min and maintained at a maximum pressure of 3 mmHg. A left posterolateral type A diaphragmatic defect of 5 cm diameter was performed using a 3 mm scissor. Each patch was placed intra-thoracic via a 5 mm port and fixed using non-absorbable silk 4/0 or 5/0 simple interrupted sutures. While placing the dHCM and Gore-Tex® patches, care was taken to place the previously marked side facing the abdominal cavity, since it has a lower pore size intended to minimize tissue attachment. Patches were tailored to the size and shape of the diaphragmatic defect. Two dHCM membranes were used in each pig. The dHCM patch was sutured to a plastic o-ring, allowing the membrane to unfold when inside the thorax and adjust to the defect area (Figure VII-2E). After suture of dHCM to diaphragmatic rims, the o-ring was removed (Figure VII-2F). Then a second dHCM membrane, also sutured to a plastic o-ring was placed above the first one (Figure VII-2G), its sutures were intercalated (Figure VII-2H) and the o-ring was removed (Figure VII-2H). Trocar-placement wounds were closed in layers using 2/0 polyglactine suture (Vicryl®; Ethicon, New Jersey, USA), and a running subcuticular 4/0 polyglecaprone suture (Monocryl®; Ethicon). Wounds were covered with a t-shirt to prevent scratching and biting during the post-operative period.

VII-2.6. Post-operative care

All animals were monitored in the operative room until the occurrence of righting reflex. Antibiotic coverage was provided by an 8-day course of enrofloxacin (5 mg/kg; IM). Post-operatively, animals were

closely checked for any signs of complication, distress, behavior changes, anorexia, or weight loss. When any of these signs was present, human endpoints were applied in order to avoid suffering.

VII-2.7. Euthanasia and necropsy evaluation

Pigs were euthanized after a postoperative period of 7 weeks. Before euthanasia, all animals underwent a thoracoscopic and laparoscopic exploration in order to evaluate adhesion formation and the macroscopic condition of the patches. Euthanasia was performed by administration of an overdose of pentobarbital (2000 mg/kg, IV) after administration of ketamine (20 mg/kg, IM) and xylazine (2 mg/kg, IM). In each animal, both hemidiaphragms were collected for further histologic evaluation. The right hemidiaphragm was used as control. In the patch repair groups, 6 tissue samples were collected from the left diaphragm: 3 in the former patch area (lesion zone) and 3 in the patch-diaphragm interface area (transition zone). Additionally, 6 tissue samples were collected from the right hemidiaphragm: 3 from the muscular area and 3 from the tendon area of the diaphragm. Same procedure was applied the Sham group.

VII-2.8. Outcome measurements

Pre- and post-operative data were collected, such as date of surgery/euthanasia, weight at surgery/euthanasia, intraoperative time, post-operative complications and/or recurrent herniation, rib synostosis, scoliosis, or wall deformation, and intrathoracic and intrabdominal adhesion formation. A scoring system for newly formed diaphragmatic tissue was adapted from Gonzalez, *et al.* [16]. Adhesion formation was assessed by thoracoscopy and laparoscopy at euthanasia and during necropsy and graded accordingly. Inflammation, angiogenesis, and fibrosis were scored through Hematoxylin-eosin (H&E) and Masson's trichrome (MT) slides (Table VII-1).

Table VII-1 Scoring system

Score	Adhesion formation	Inflammation	Angiogenesis	Fibrosis
0	None	None	None	None

1	Minimal adhesions that could be freed by gentle blunt dissection	Minimal	Minimal	Minimal
2	Moderate adhesions that could be freed by aggressive blunt dissection	Mild	Mild	Mild
3	Dense adhesions that required sharp dissection	Moderate	Moderate	Moderate

VII-2.9. Histology

Samples were fixed in 10% formalin, embedded in paraffin, and cut in 5 µm sections. H&E and MT staining were performed. At least five fields per slide were randomly analyzed and scored (Table 1) by one operator (AL-F) who was blinded to the study groups and outcome at the time of the evaluation. An Olympus System Microscope was used to photograph the stained section.

VII-2.10. Immunohistochemistry

Paraffin-embedded sections were incubated with rabbit anti-alpha smooth muscle actin (anti- α SMA; 1:50, #ab5695; Abcam, Cambridge, UK), rabbit anti-CD31 (1:50, #ab28364; Abcam) and mouse anti-CD105 (1:100, #MCA1557F; BioRad, California, USA) for 2 h at RT. A secondary antibody from the R.T.U. VECTASTAIN® Universal ABC Elite® Kit (#PK-7200, Vector Laboratories, UK) was used in accordance with manufacturer's instructions. Incubation was revealed using Peroxidase Substrate Kit (DAB) (#SK-4100, Vector Laboratories). Sections were counterstained with hematoxylin and mounted in an aqueous mounting medium. Slides were observed in an optical microscope with a coupled camera (DM750, Leica, Wetzlar, Germany).

VII-2.11. Statistically analysis

GPower version 3.1.9.2 (program written by Franz Faul, Universität Kiel, Germany 1992-2014) was used to calculate the minimum number of animals (effect size 0.7), to obtain a statistical power of 95%. Results

analysis was performed using GraphPad Prism 7 (GraphPad Software, Inc. 2016). Comparisons between groups were performed by One-way ANOVA followed by Dunn's multiple comparison test. To compare the survival distributions of the groups, Logrank test was used. A $p < 0.05$ was considered statistically significant.

VII-3. RESULTS

VII-3.1. dHCM is associated with higher surgical time

Thoracoscopic defect creation and diaphragmatic repair was performed without any technical difficulty in all groups (Figure VII-2). One animal (dHCM group) died during surgery due to anesthetic complications and was excluded from the study ($n = 13$). Overall surgery time was 178.9 min. However, intraoperative time (Figure VII-3A) was higher in the dHCM (240.0 min) than in the PCLem (172.5, $p = 0.203$) and Gore-Tex® (156.3 min, $p = 0.025$) groups. During the surgeries, 3 pigs (G3, P2, P3) presented intrathoracic signs of previous pneumonia, such as adhesions and mucus. Nevertheless, those issues did not influence surgery duration (Figure VII-3A). One pig (P3) presented respiratory distress at the end of the surgery due to aspiration, as well difficulty in left foreleg locomotion in the postoperative period (probably due to brachial plexus lesion during surgery). No other types of intra- or post-operative complications occurred.

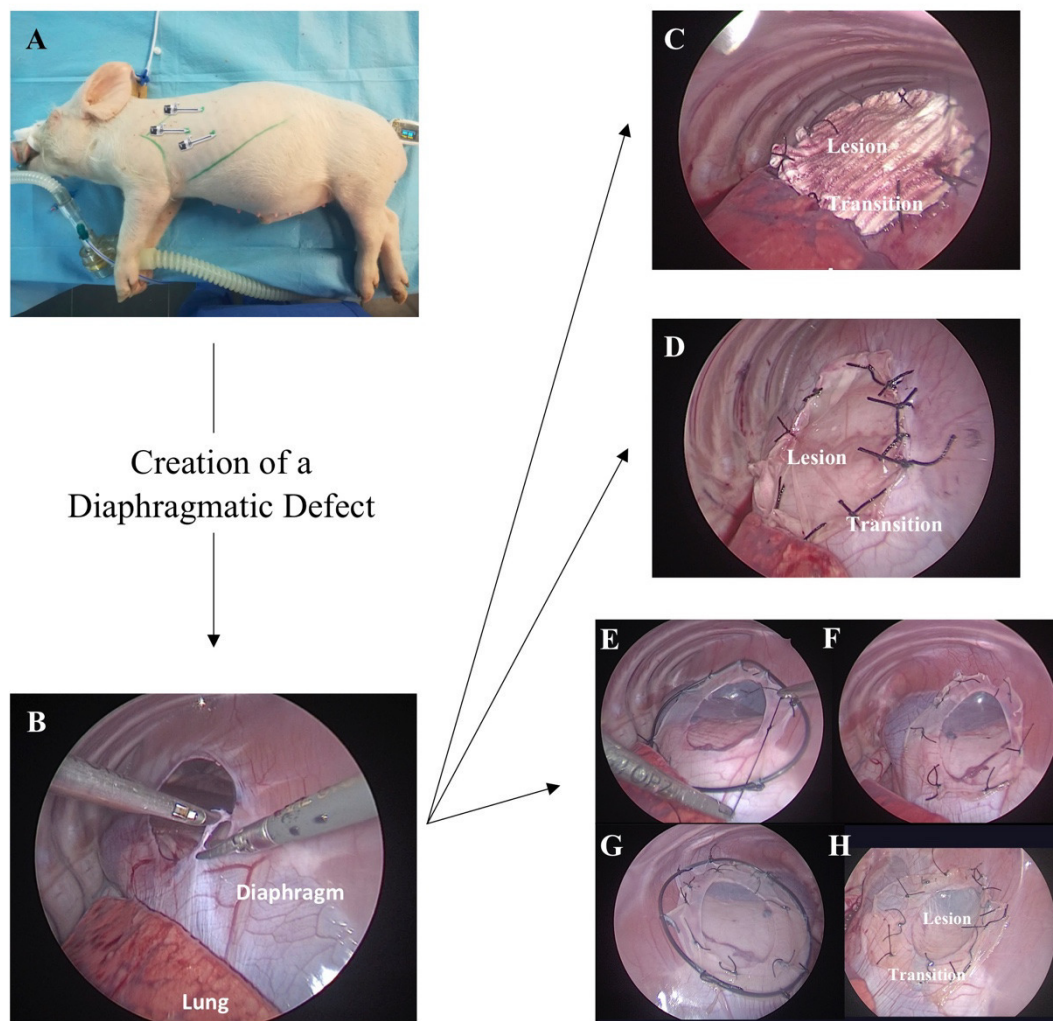


Figure VII-2 Thoracoscopic defect and repair of the diaphragm. Representation of trocars site (A). Thoracoscopic creation of a diaphragm defect (B). Repair of the diaphragm defect with Gore-Tex® (C), PCLeM (D) and dHCM (E-H).

VII-3.2. PCLeM and dHCM are associated with hernia recurrence

From the total 13 animals included in this study, 8 animals (2 from Sham, 4 from Gore-Tex® and 2 from PCLeM groups) survived until the end of the experimental timepoint of 7 weeks. Five animals – 3 from dHCM and 2 from PCLeM groups – developed sudden dyspnea and anorexia 14, 23, 27, 33, and 45 days after surgery, respectively, and humane endpoints were applied. A complete or partial detachment of the patch and associated herniation of the stomach into the left hemithorax was found during necropsy. Concerning the animals that were not submitted to humane endpoints, chest x-ray performed at the end of the follow-up period (Figures VII-3C-E) revealed a normal diaphragm, with no

evidence of eventration, hemithorax volume reduction, lung pathology, scoliosis or chest wall deformities when compared to pre-operative imaging (Figure VII-3B). The hernia recurrence rate was 0% in Gore-Tex® group, 50% in PCLem group and 100% in dHCM group (Figure VII-3A). These results were reflected in follow-up time and weight gain of the animals (Figure VII-3A). For the animals that make it to 7 weeks (S1, S2, G1, G2, G3, G4, P2, P3), the overall weight gain was 27.4 kg, corresponding to a triplication of the original weight (mean: 8.9 kg, data not shown). As previously stated, at the time of euthanasia all animals underwent thoracoscopy and laparoscopy. Although thoracic scars were unremarkable, 5 animals (3 from Gore-Tex® - G2,G3,G4 - and 2 from PCLem -P2, P4- groups) presented postoperative calcification adjacent to the 6th, 7th and 8th ribs, where trocars were placed (data not shown). Regarding adhesion formation, though no statistically significant differences were observed between the 3 treatment groups ($p = 0.121$), PCLem and Gore-Tex® groups had a similar mean score of 2.3, while dHCM group was associated with a lower level of adhesion formation (mean score of 1.3), similar to the one observed on sham group (mean score 1.5) (Figure VII-3A). Gore-Tex® was associated with adhesions to the lung and the omentum, although the smooth side of the dual mesh was correctly placed facing the abdominal cavity in order to prevent such complication. PCLem group presented gastric adhesions and spread and tenacious adhesions both in thorax and abdomen. dHCM adhesions were minimal and limited to trocar level. In sham group, adhesions were only observed at thoracic incision points and between lung and diaphragm.

A

Experimental Groups	Animals	Surgery Duration (min)	Surgery Duration (min) mean	~7 weeks follow up	Follow up time (days)	Recurrence Rate (%)	Weight gain (Kg)	Adhesions grade (score 0-3)	Adhesions grade (score 0-3) mean
Sham group (n=2)	S1	150	145.0	Yes	52	0	21.9	2	1.5
	S2	140		Yes	51		24.7	1	
Gore-Tex® group (n=4)	G1	130	156.3	Yes	50	0	34.6	3	2.3
	G2	180		Yes	47		21.2	2	
	G3	150		Yes	48		31.2	2	
	G4	165		Yes	63		36.1	2	
PCLem group (n=4)	P1	180	172.5	No	45	50	15.9	2	2.3
	P2	150		Yes	47		29.4	2	
	P3	180		Yes	48		19.9	3	
	P4	180		No	33		11.4	2	
dHCM group (n=3)	D1	300	240.0	No	14	100	6.1	1	1.3
	D2	210		No	27		10.0	1	
	D3	210		No	23		9.4	2	

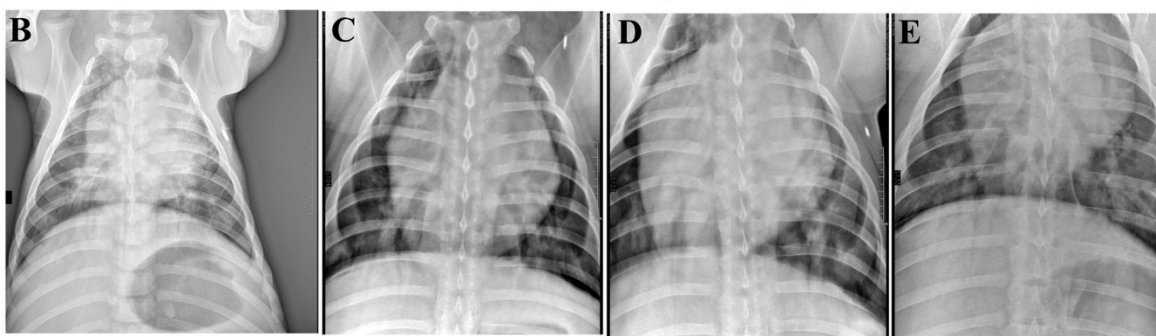


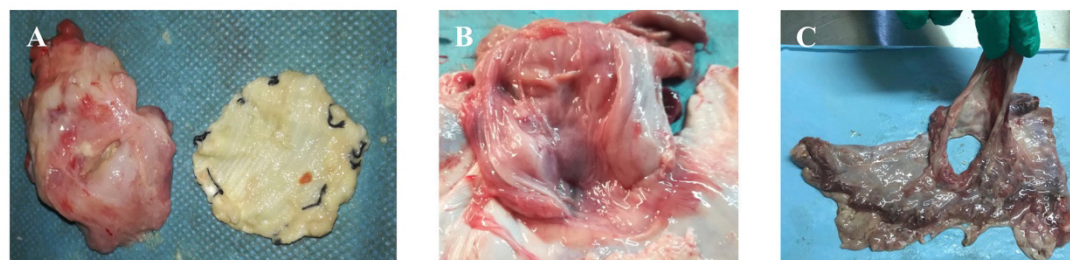
Figure VII-3 Outcome Measurements. Descriptive table summarizing different outcome measures, such as surgery duration (min), follow up time (days), recurrence rate (%), weight gain from surgery to euthanasia (kg), and adhesion formation grade at time of death per animal **(A)**. Representative chest x-ray image prior to **(B)** and post-surgery, at time of euthanasia, in Sham **(C)** Gore-Tex® **(D)**, and PCLem **(E)** groups. Since all animals of dHCM group were submitted to humane endpoints, no chest X-rays were performed post-surgery.

VII-3.3. PCLem and dHCM are associated with better tissue integration

At euthanasia, Gore-Tex® patches appeared virtually unchanged and covered with a fibrotic capsule (Figure VII-4A). The thoracic surface of the implanted patch was partially agglutinated to the lung in 3 animals (G1, G2 and G4), and the abdominal surface was agglutinated to the omentum in all animals ($n = 4$). Moreover, one animal (G3) presented serous fluid accumulation between the layers of the regenerated fibrotic tissue. Another animal (G1) had intrathoracic signs of infection/inflammation and a capsulated abscess in the area of Gore-Tex® implantation. Nevertheless, this animal never presented clinical signs of infection during the follow-up period, presenting a weight gain of 34.6 kg, higher to the mean of Gore-Tex® group (30.8 kg) and reflecting an initial body weight quintuplication in 50 days. In contrast, in the PCLem and dHCM groups, the patches could not be identified. In the PCLem group, it was replaced by a floppy connective tissue (Figure VII-4B). In both animals submitted to humane

endpoints (P1 and P4), adhesions between lung, patch and stomach were observed. Regarding the other two animals in the PCLem group (P2 and P3), one presented partial agglutination between the thoracic surface of the implanted patch and the lung (P2), while the second had dense adhesions between abdominal organs (peritonitis), a bulging stomach and compact adhesions between lung, patch and stomach (P3). Interestingly, no weight loss was observed in this last animal, presenting a weight gain of 19.9 kg which corresponds to a 200% increase from the animal initial weight (10.1 kg). In the dHCM group, the patch was replaced by a vascularized and floppy regenerated membranous tissue (Figure VII-4C). One pig (D3) presented the thoracic surface of the patch partially agglutinated with the lung.

After macroscopic analysis, H&E and MT staining were performed to analyze inflammation, angiogenesis, and fibrosis grade. As previously described, a scoring system was used (Table VII-1). A summary of the results is presented in Figure VII-4D. Although no statistically significant differences were observed ($p = 0.273$), inflammation was slightly lower in the lesion site in the Gore-Tex® group (Figure VII-4D). This slight decrease in inflammation grade was also observed in the transition zone in Gore-Tex® and PCLem groups in comparison to dHCM group, which had the higher score for inflammation in both sites (lesion and transition). Inflammation scores did not change from lesion to transition site in Gore-Tex® and dHCM groups. In PCLem group a decrease was observed between the two sites. A lymphoplasmacytic inflammation was observed in all groups with the presence of some neutrophils, macrophages, and foreign-body giant cells. Regarding angiogenesis (Figure VII-4D), no statistically significant differences were observed in the lesion site, although dHCM group presented a lower score. In the transition zone, dHCM group had the higher score for angiogenesis and a statistically significant difference was observed when compared with Gore-Tex®, where angiogenesis was not observed ($p = 0.037$). While PCLem and dHCM groups shown an increase in the score from lesion to transition site, in the Gore-Tex® group a decrease is observed. This decreasing trend from lesion to transition site was maintained for fibrosis grade in Gore-Tex® and PCLem groups, in contrast to the dHCM group that shows an increase (Figure VII-4D). At the lesion site, fibrosis grade is higher in PCLem group and lower in dHCM group. However, in the transition zone, dHCM group shows the highest score and Gore-Tex® group the lowest. Nevertheless, no statistically significant differences were observed for fibrosis in both tissue zones (lesion and transition).



D		GoreTex group (n=4)	PCLem group (n=4)	dHCM group (n=3)	P value
Inflammation grade	Lesion	2.5	3.0	3.0	0.273
	Transition	2.5	2.5	3.0	>0.999
Angiogenesis grade	Lesion	2.0	2.0	1.3	0.844
	Transition	0.0 *	2.3	3.0 *	0.030
Fibrosis grade	Lesion	1.5	2.5	1.3	0.662
	Transition	0.8	1.5	3.0	0.247

* $p = 0.037$

Figure VII-4 Macroscopic and microscopic evaluation of new diaphragmatic tissue. Top view of Gore-Tex® (A), PCLem (B) and dHCM (C) after follow-up time. Comparison of inflammation, angiogenesis and fibrosis mean scores (score 0-3) between groups, defined by the histological analysis of H&E and MT staining. Two different tissue areas were analyzed, lesion site that corresponds to the patches and transition that represents the native diaphragm at the site of the sutures (D).

To further assess the tissue regeneration, immunohistochemistry was performed for muscle (α -SMA), endothelium (CD31) and stem cells (endoglin; CD105) (Figure VII-5). A positive staining for α -SMA was observed in all groups (Figure VII-5C-H). However, this staining was more intense in the Gore-Tex® group (Figure VII-5C and 5D) and less intense in the PCLem group (Figure VII-5E and VII-5F). In all conditions, the transition zone (Figure VII-5D, VII-5F and VII-5H) presented a higher staining for α -SMA when compared to the lesion zone (center). Moreover, α -SMA-positive blood vessels were observed in dHCM in both zones. Regarding CD31 staining, although it was present in the three groups in both zones (lesion and transition; Figure VII-5K-P), it was more intense in dHCM transition zone (Figure VII-5P).

CD105 positive staining was only observed in inflammatory infiltrates, regardless patches and zones (lesion or transition; Figure VII-5S-X).

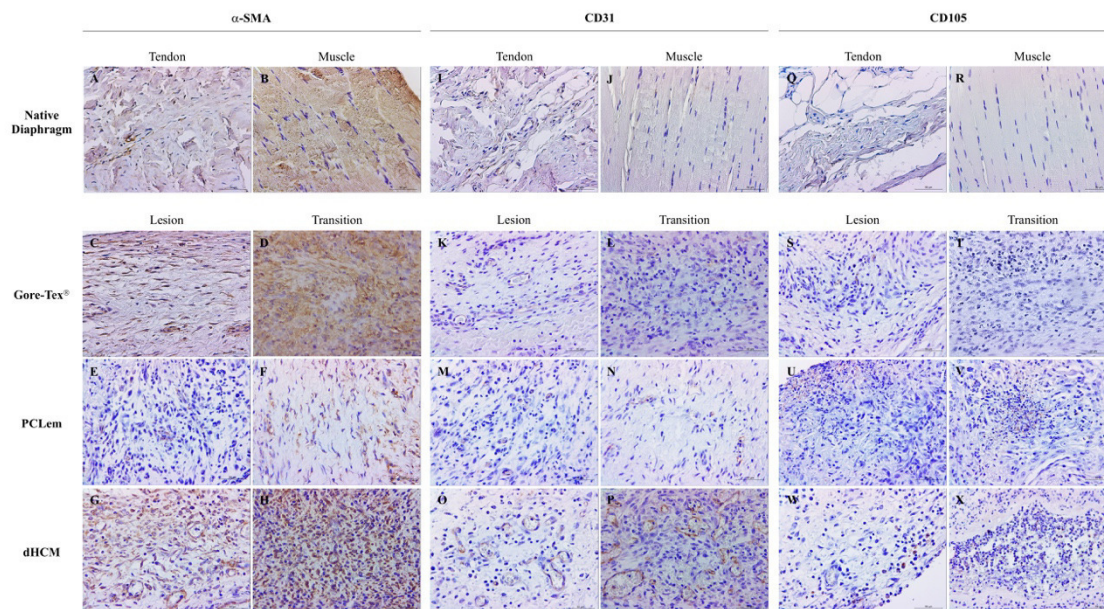


Figure VII-5 Immunohistochemistry for α -SMA, CD31 and CD105. Representative sections of α -SMA, CD31 and CD105 staining of the native diaphragm, in both tendon (**A, I and O**, respectively) and muscle (**B, J and R**, respectively). Patch samples were divided into two distinct zones: lesion and transition (Figure VII-2C, VII-2D and VII-2H). Representative sections of α -SMA, CD31 and CD105 for Gore-Tex® (**C-D, K-L, S-T**, respectively), PCLem (**E-F, M-N, U-V**, respectively) and dHCM (**G-H, O-P, W-X**, respectively) groups.

VII-4. DISCUSSION

In this study, a novel diaphragmatic hernia animal model was implemented for the first time, using thoroscopic techniques in sexually immature (6 weeks) farm pigs. Rounded diaphragmatic defects with a 5 cm diameter were created in order to mimic large diaphragmatic defects and to allow post-implantation clinical evaluation [1]. Since this diaphragmatic defect would be fatal and uncomfortable to live with, sham animals were only submitted to thoracoscopy without creation of the defect. Sham animals survived until the end of the study without any complication, validating the surgical technique. This animal model is associated with a rapid growth rate that would be identical to the human newborn. Although this growing animal model is frequently found in clinical settings of open surgery [16,32], the hypoplastic lung parenchyma characteristic of CDH is absent. Moreover, in this study, diaphragm rims

were maintained to allow patch placement (A defect), excluding the clinical cases of CDH where the patch is needed (large B, C and D defects) [1]. Nevertheless, this model gives insights on how new membranes would behave in a thoracoscopic technique and in diaphragmatic hernia repair, opening new research lines for CDH.

As it would be expected for animals that have been bred predominantly for meat production, pigs' musculature was massive. Prior to this study, a pilot study with 7 animals (1 Sham, 2 Gore-Tex®, 2 PCLem and 2 dHCM) was performed to allow the surgeon to become familiar with the handling properties of each material and to perfect both operative technique and post-operative management. This allowed for surgeries to be performed without any technical difficulty in all groups (Gore-Tex®, PCLem and dHCM). However, dHCM group was associated with a higher surgery time, due to the handling of dHCM fixed in o-rings and to the use of two membranes per animal. Without o-rings it was impossible to stretch dHCM in the thoracoscopic space. Moreover, to guarantee the closure of the diaphragmatic hernia, two dHCM had to be used per pig. During this study, the overall pig's weight gain was 20.5 ± 10.9 kg in 42.2 ± 13.8 days, being in accordance with farm-pigs growth curve [41]. Adhesions are a result of the inflammatory response to injury in the peritoneal space [42]. The grade of the adhesions varies in accordance with the type of patch implanted. The level of adhesions of the dHCM group was similar to that of the Sham group. In contrast, Gore-Tex® and PCLem groups presented a higher level of adhesions. Moreover, considering that all surgeries were performed by the same surgeon in the same conditions, it was hypothesized that the spread and severity of the adhesions, both in the thorax and the abdomen, of the PCLem group may be due to a foreign body inflammatory response elicited by the synthetic patches [42]. As longitudinal follow-up measurements, chest x-rays were performed before patch implantation and at the end of the study. All animals had the same amount of tissue excised, making all repairs comparable. Moreover, no musculoskeletal sequelae were found, probably due to the small rim of muscle left during the diaphragm excision, which could impact the ingrowth of the patch and reduce the tension on the chest wall.

Diaphragm manipulation with prothesis implantation is always necessarily followed by an inflammatory process that ultimately leads to inclusion, degradation, or rejection of the prothesis. As foreign bodies, all patches will trigger a process of inflammation, fibrosis, and neovascularization [43,44]. No significant differences in inflammation grade were observed between the three groups (Gore-Tex®, PCLem and dHCM). Nevertheless, a slight decrease in inflammation score was observed in Gore-Tex® and PCLem groups when compared with dHCM, which may be explained by an inflammation resolution over time associated with the higher follow-up times of these groups. As expected [18], a

lymphoplasmacytic inflammation with the presence of some neutrophils, macrophages, and foreign-body giant cells was observed in all conditions. In general, fibrosis grade was comparable between the three groups. However, a slight but not statistically significant difference was observed between Gore-Tex® and dHCM groups in the transition zone. Similar results had been reported in studies using Gore-Tex® and Surgisis® [16,17]. As previously reported [16–18], Gore-Tex® presented poor tissue integration and its surface was covered by a fibrotic capsule which was likely due to a foreign body response. In contrast, dHCM was replaced by a vascularized connective tissue, preventing the identification of the membrane, as already described for natural membranes [16,17]. On the other hand, the surface of PCLem was replaced by a floppy connective tissue and the membrane was no longer identifiable, neither macro nor microscopically. This was not in accordance with the results of Zhao *et al* [29] that used aligned PCL/collagen type I hybrid scaffolds for diaphragmatic muscle reconstruction. In that study, the scaffold was identifiable until the 4th month (approximately 112 days). Nevertheless, Zhao *et al* [29] used a different animal model (female Lewis rats with 14 weeks-old) which may influence the degradation rate of the material [29]. As previously described [24], the center of the patch (lesion zone) was relatively free of α -SMA positive cells when compared to the periphery (transition zone). Moreover, α -SMA positive blood vessels were observed in dHCM in both lesion and transition zones, suggesting a functional tissue vascularization at 21.3 ± 6.7 days post-surgery. In fact, a higher number of CD31-positive cells (endothelial marker) were observed in dHCM in both zones when compared with Gore-Tex® and PCLem. This was also corroborated by the angiogenesis scores. By its side, the PCLem group presented the lowest CD31 staining, although it also presented the higher CD105 staining intensity, suggesting a neovascularization preparation [45–47]. So, in the PCLem group, neovascularization is expected to occur after 43.3 ± 7.0 days (6 weeks). This was already observed in a similar study using aligned PCL/collagen type I hybrid scaffold [29], where visible graft vascularization started to be observed on the 2nd month and increased until the 6th month.

In general, PCLem and dHCM groups were associated with better tissue integration than Gore-Tex®, but also with a lesser follow-up time related with hernia recurrence (50% in PCLem and 100% in dHCM). It was hypothesized that while the patch was degraded, the replacing tissue was weaker than the native one and not able to prevent recurrence when it was subjected to tension related to the rapid growth of the animal. Thus, the pig's rapid growth curve together with fast patch degradation could have influenced the recurrence rate. This was not expected since it was demonstrated that, when placed subcutaneously in mice, dHCM only started to be integrated by host tissue at day 28 [40]. So, it is

important to understand if the rapid patch degradation is related with pig's inflammatory response and collagen metabolism or if it will persist in other animal models, and if it is related with the implantation site. In future studies more dHCM layers should be used in accordance with what is done with Surgisis® (4 to 8 ply) [16–18]. Despite no recurrent herniation was observed in the Gore-Tex® group, in 2 cases the patch was partially detached from the diaphragmatic rim.

The obtained results support the hypothesis that both PCLeM and dHCM membranes would be integrated by the host tissue and consequently accompanied the subject growth, what is not observed when Gore-Tex® is used [16–18]. Future studies with longer follow-up times using PCLeM and more layers of dHCM would be important to assess this issue.

Minimal invasive surgery, such as thoracoscopic, has been associated with higher recurrence rates when compared with open surgery [13], the benefits of one technique over the other remain inconclusive [10,13]. Regarding PTFE-based membranes (such as Gore-Tex®) a recurrence rate of 0% was observed in our study. Nevertheless, in a study using open abdomen surgery for diaphragmatic hernia treatment in Yucatan pigs, the recurrence rate of PTFE-based membranes was 30% [16]. In the same study, a natural membrane was used (SIS) that presented a recurrence rate of 0% [16] in contrast with our results (100% recurrence rate dHCM). Although this may be associated with the surgical technique it can also be related with the number of membrane layers used, as previously stated. While two dHCM layers were used in this study, in Gonzalez, *et al* 8 layers of SIS were used [16]. Another study using open abdomen surgery for the treatment of diaphragmatic hernia using cryopreserved and decellularized human hemidiaphragm in a dog model, demonstrated a herniation recurrent rate of 0% after 6 months of follow-up [48]. Nevertheless, the nature and composition of the patches is different from the ones used in this study, which may have a greater influence in the study outcomes than the surgical procedure alone.

No muscle layers were observed in the implanted areas after 40.5 ± 14.4 days. This result was already expected since muscle formation is only described to be found in implanted areas after 24 weeks (168 days) [32]. In further studies, longer follow-up times are needed to assess muscle formation. One major concern about this study is the difference between forces acting on the pig diaphragm when compared to human, since animals spend most of their time walking on “all fours”, thereby possibly influencing different stress patterns on the grafts and altered frequency of herniation.

VII-5. CONCLUSIONS

In this work, sexually immature farm pigs and thoracoscopic techniques were used to establish a new diaphragmatic hernia model. This growing large animal model was used to compare two new membranes, one synthetic (PCLeM) and another biological (dHCM) against the clinical gold standard (Gore-Tex®) for diaphragmatic hernia repair. In summary, when compared to Gore-Tex®, PCLeM and dHCM were associated with better tissue integration and neovascularization (in preparation for PCLeM and functional in dHCM), suggesting that the patches would grow with the subject. Nevertheless, PCLeM and dHCM were also related with a higher hernia recurrence (50% and 100%, respectively), when compared to Gore-Tex® (0% recurrence rate). Additional long-term studies using PCLeM and more layers of dHCM would be important to create a solution that allows tissue regeneration *in situ* without hernia recurrence. Moreover, improvements in animals' models and membrane design are needed.

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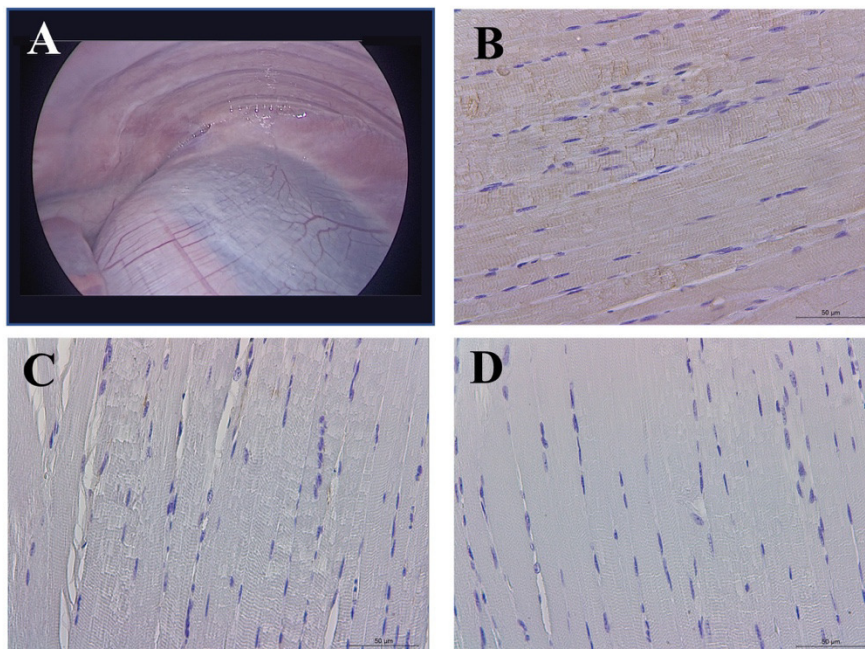
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VII-9. SUPPLEMENTARY MATERIAL



Supplementary Figure VII-1 Sham animals' characterization. Thoracoscopic sham surgery without defect creation **(A)**. Representative sections of α -SMA **(B)**, CD31 **(C)** and CD105 **(D)** staining of diaphragm section from sham animals.

SECTION 6

GENERAL CONCLUSIONS

Chapter VIII

General Conclusions and Future Perspectives.

Chapter VIII

General Conclusions and Future Perspectives.

VIII-1. GENERAL CONCLUSIONS

In this thesis we validated the hypothesis of using the decellularized human chorion membrane as a new biomaterial. The decellularization protocol of the human chorion membrane was optimized and described for the first time. The resulting membrane was characterized and tested for biomedical applications both *in vitro* and *in vivo*. Altogether the obtained results support the use of dHCM as a new biomaterial that was also patented by us.

The dHCM is a compact and easy to handle membrane with two different surfaces (reticular layer side and trophoblast layer side) with different topographies and composition. In general, dHCM trophoblast side was associated with a slightly higher cell metabolic activity when cell lines were cultured *in vitro* which may be related with the presence of the basement membrane proteins (collagen type I and IV, fibronectin, and laminin). On the other hand, dHCM reticular layer showed a stronger biological performance when seeded with a primary cell line (HUVEC). This dHCM side also demonstrated a lower blood protein adsorption and a slightly higher anti-thrombogenic activity. No differences were observed between both dHCM sides regarding its angiogenic (CAM assay) and antibacterial (against *S. aureus*) activity.

dHCM properties allowed us to explore its use in three different biomedical applications (section 4), the creation of *in vitro* models (Chapter V), vascular grafts (Chapter VI) and as a surgical patch (Chapter VII). Firstly, a functional BBB *in vitro* model was created using dHCM to mimic the BBB basement membrane. Our dHCM-based BBB *in vitro* model showed higher TEER values than others described in the literature. Moreover, our results suggest that three different transports routes across the BBB (membrane diffusion, solute carrier, and receptor-mediated) were functional. Secondly, it was demonstrated that the dHCM has properties compatible with those of small caliber vascular grafts. A dHCM tube with 3-4 mm in diameter was fabricated with the reticular layer side facing the inner side of the tube and its mechanical properties were in the range of the ones of the saphenous vein (the gold standard for autologous small diameter vessel replacement). Finally, the dHCM was used as a surgical

patch for diaphragmatic repair in a growing large animal model (*Sus scrofa domestica*). dHCM was associated with a better tissue integration and neovascularization when compared with Gore-Tex (the gold standard for diaphragmatic hernia repair). Nevertheless, it was also associated with a higher hernia recurrence rate (100%) which may be eventually solved by using more than two dHCM layers.

Altogether, the set of works developed in this thesis, allowed obtaining a detailed characterization of the dHCM for the first time. It was demonstrated that the dHCM has unique characteristics that make it a valuable new biomaterial that was validated herein for some biomedical applications both *in vitro* and *in vivo*. dHCM has promising properties that make it a strong candidate for many other biomedical applications.

VIII-2. FUTURE PERSPECTIVES

The findings of this thesis allowed for a deeper understanding of using decellularized inner body membranes as biomaterials, focusing on dHCM. Although dHCM's composition and properties were characterized, further studies are needed to fully validate its use in the clinic. Moreover, the application of dHCM in different tissue engineering areas such as bone, cartilage, tendon, skin, and ocular regeneration was not investigated.

Further drug screening studies should be performed using the BBB *in vitro* model developed using dHCM, in order to fully validate the model application. Moreover, based on the developed model, disease models of stroke or traumatic brain injury could be developed envisioning its use to study disease's mechanisms and to establish and test strategies to accelerate BBB repair. These additional works would reinforce the use of this dHCM-based BBB *in vitro* model as a valid and representative alternative or complementary strategy to animal testing.

dHCM *in vivo* behavior was investigated subcutaneously (in mice) and in the thoracic space for hernia regeneration (in a new pig model). Nevertheless, due to the different inflammatory responses and collagen metabolism associated with the implantation site and different animals' metabolisms, dHCM degradation and integration by host tissue occurred at different rates. Subcutaneously in mice, dHCM started to be integrated by host tissue at day 28, while in pigs this occurred around the third week (21 days). So, it would be important to verify if the degradation rate of dHCM vascular graft *in vivo* would match the rate of vascular tissue regeneration, avoiding aneurysms or vascular rupture, fully validating its functionality. Moreover, in future studies, regarding hernia treatment, more dHCM layers should be used to increase its mechanical properties, following a similar strategy to that used with SIS.

The work developed in this thesis was focused on the characterization and application of dHCM alone. Nevertheless, there are several studies combining decellularized inner body membranes with synthetic polymers. This could be a research line to be followed in order to increase dHCM mechanical properties and decrease its *in vivo* degradation rate. Moreover, decellularized inner body membranes have been used as substrates to support cell delivery which was not investigated using dHCM. Overall, dHCM is a novel biomaterial and there are a lot of research questions and hypothesis that were not answered and validated yet.