

Engineering Vascularized Microenvironments to Enhance Epithelial Organoid Complexity

Iasmim Diniz Orge

D
2025



Iasmim Diniz Orge
Engineering Vascularized Microenvironments to Enhance
Epithelial Organoid Complexity

D. ICBAS 2025



Engineering Vascularized Microenvironments to Enhance

Epithelial Organoid Complexity

Iasmim Diniz Orge

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR



PhD Thesis

Engineering Vascularized Microenvironments to Enhance Epithelial Organoid Complexity

Iasmim Diniz Orge

Thesis presented to the International Doctoral Program in Molecular and Cellular
Biotechnology applied to Health Sciences (BiotechHealth) of the University of Porto

2025

Iasmim Diniz Orge

Engineering Vascularized Microenvironments to Enhance Epithelial Organoid Complexity

Thesis submitted to the University of Porto (U: Porto) - the Abel Salazar Institute of Biomedical Sciences (ICBAS UP) and the Faculty of Pharmacy

This thesis was supervised by:

Supervisor: Cristina C. Barrias, PhD

Affiliation: Instituto de Investigação e Inovação em Saúde (i3S), Instituto de Engenharia Biomédica (INEB), and Instituto de Ciências Biomédicas Abel Salazar (ICBAS) da Universidade do Porto.

Co-Supervisor: Pedro M. Baptista, PhD.

Affiliation: Health Research Institute of Aragon (IIS Aragon), Spain.

Co-Supervisor: Silvia Bidarra, PhD.

Affiliation: Instituto de Investigação e Inovação em Saúde (i3S), Portugal

HOST INSTITUTIONS

The research described in this thesis was conducted at Instituto de Investigação e Inovação em Saúde (i3S) and at Health Research Institute of Aragon (IIS Aragon).

FUNDING

This work was supported by Portuguese funds via FCT – Fundação para a Ciência e Tecnologia, in the framework of project EndoSWITCH (PTDC/BTM-ORG/5154/2020).

Additionally, this research was funded by the REMODEL project from European Union's Horizon 2020 Research and Innovation Program under grant agreement number 7857491. Iasmim Orge was a recipient of FCT scholarship: SFRH/BD/ 2020.07458



REPÚBLICA
PORTUGUESA

CIÊNCIA, TECNOLOGIA
E ENSINO SUPERIOR



UNIÃO EUROPEIA

Fundo Social Europeu
Iniciativa Emprego Jovem



European
Commission



REMODEL

ACKNOWLEDGEMENTS

Firstly, I would like to acknowledge myself. For being insufferably persistent and sticking to all the difficult choices I had to make to be able to see this thesis successfully completed. During these 4 years (actually 3 and ½ years), I had to face scientific and personal challenges that shaped my personality and maturity as a researcher and a person, and I'm happy to have never given up on what I believed it was right to explore, and at the same time had the wisdom to let go of what couldn't be fixed.

Secondly, I'd like to thank my family for all the support, especially my fiancé Bruna Zurita—for all the times she helped me get up and face the problems when all I wanted to do was run. Thank you to my parents, Luiz and Soraya, for your unconditional support and for always reminding me that I could go further, and to my friends, near and far, for never stopping believing in me (even when I sounded a little unhinged).

Of course, I also want to acknowledge my supervisors—especially Cristina Barrias, for being such an inspiring scientist, always open to discussing even my wildest project ideas, and for contributing brilliant ideas of her own that made this thesis so much more exciting. Thank you for your patience through the bureaucratic labyrinth at the beginning of my PhD, and for never giving up on me. I also want to thank my co-supervisor Pedro Baptista for being the ideal “liver boss” and a constant source of encouragement, support, and big-picture perspective.

Thanks to all the incredible BEMIC group members for being more than colleagues—thank you for the great lunch company, post-lab beers, spontaneous laughs, sunsets, and of course, for all the help in the lab. You made even the long days bearable (and occasionally fun!).

And a special shout-out to Seb and Gui—my beloved (and now dearly missed) hamsters—for being my tiny, furry anchors. You greeted me every late night when I came home, reminding me that work is not everything.

Lastly, I want to thank the science itself—for being hard, unpredictable, humbling, and completely worth it.

“This is ten percent luck, twenty percent skill, fifteen percent concentrated power of will... five percent pleasure, fifty percent pain, and a hundred percent reason to remember the name.”

— Fort Minor, Remember the Name

ACHIEVEMENTS

PAPERS

Orge, ID; Ferreira SA; Barrias CC. Vascular units as living building blocks for bottom-up tissue engineering. To be submitted for publication as a review paper.

Orge, ID; Blanes A; Nascimento DS; Souza B; Baptista PM; Barrias CC. A peri-bile ductal fibrovascular stroma-on-chip for dissecting multicellular interactions in an inflammatory and fibrogenic liver microenvironment. To be submitted for publication.

Orge, I. D.; Nogueira Pinto, H.; Silva, M. A.; Bidarra, S. J.; Ferreira, S. A.; Calejo, I.; Masereeuw, R.; Mihăilă, S. M.; Barrias, C. C., Vascular units as advanced living materials for bottom-up engineering of perfusable 3D microvascular networks. *Bioact Mater* 2024, 38, 499-511.

COMUNICATIONS

Orge, ID; Blanes A; Baptista PM; Barrias CC. A Vascularized Bile Duct-on-a-Chip Platform to culture Intrahepatic Cholangiocyte Organoids. TERMIS European Chapter Meeting 2025. Freiburg, Germany, 2025. Oral presentation.

Orge ID, Reis R, Maia A, Bidarra SJ, Barrias CC. A microtissue-engineering strategy for generating stromal-vascular networks on-chip. EMBO Workshop Building networks 2024: Engineering in vascular biology. Barcelona, Spain. 2024. Poster presentation.

Orge ID, Pinto HN, Silva M, Calejo I, Bidarra SJ, Mihaila SM, Masereeuw R, Barrias CC. Perfusable vascularized stroma on-chip for growing 3D organotypic structures. TERMIS European Chapter Meeting 2023. Manchester-UK, 2023. Oral presentation.

Orge ID, Pinto HN, Silva M, Calejo I, Bidarra SJ, Mihaila SM, Masereeuw R, Barrias CC. Perfusable vascularized stroma on-chip for growing 3D organotypic structures. 2nd MPS World Summit. Berlin, Germany, 2023. Poster presentation.

Orge ID, Pinto HN, Silva M, Calejo I, Bidarra SJ, Mihaila SM, Masereeuw R, Barrias CC. Vascular units as advanced living materials for bottom-up engineering of perfusable 3D microvascular networks. Spanish & Portuguese Advanced Optical Microscopy Meeting. Faro, Portugal. 2023. Oral presentation.

Orge ID, Pinto HN, Silva M, Calejo I, Bidarra SJ , Mihaila SM , Masereeuw R , Barrias CC. Perfusable vascularized stroma on-chip for growing 3D organotypic structures. Beyond REMODEL: 3R models for biomedical research. Porto, Portugal, 2023. Poster presentation.

Orge ID, Pinto HN, Silva M, Calejo I, Bidarra SJ , Mihaila SM , Masereeuw R , Barrias CC. Bioengineered microfluidic chamber for organoid vascularization. REMODEL Summer School: Next-Generation Organoids and Organ-on-a-chip. Porto, Portugal, 2022. Oral presentation.

TABLE OF CONTENTS

ABSTRACT	16
RESUMO	17
ABBREVIATIONS.....	19
THESIS OUTLINE.....	22
SECTION A - GENERAL INTRODUCTION	24
Chapter I.....	26
1. Microvascular engineering approaches.....	28
2. Biofabrication of VUs	30
2.1. <i>Methodologies for VUs formation</i>	30
3. Intrinsic factors affecting VUs structural and functional properties.....	31
3.1. <i>Cell sources</i>	32
3.2. <i>Cell composition</i>	34
4. Extrinsic factors affecting VUs structural and functional properties	37
4.1. <i>Hydrogel matrices</i>	38
4.2. <i>Biochemical cues</i>	42
5. Integrating VUs into advanced systems	43
5.1. <i>VUs assembly into larger constructs via direct fusion</i>	43
5.2. <i>VUs assembly using 3D printing</i>	46
6. VUs as advanced tools for drug testing.....	54
7. VUs for tissue repair in trauma and disease.....	56
8. Challenges, industrial outlook and Clinical translation	58
9. Conclusions and future outlook.....	59
Chapter II.....	70
1. The need for vascularization in organoid cultures	72
2. Intrahepatic cholangiocyte organoids	75
3. Vascularized models of intrahepatic cholangiocyte organoids.....	76
MOTIVATION AND AIMS	83
SECTION B - EXPERIMENTAL RESULTS	85
Chapter III.....	87
ABSTRACT	89
1. Introduction.....	91
2. Materials and Methods.....	93
2.1. <i>Vascular units (VUs)</i>	93
2.2. <i>Microfluidic device</i>	95
2.3. <i>Organoid culture</i>	97
2.4. <i>Image analysis</i>	98
2.5. <i>Statistical analysis</i>	98
3. Results.....	99
3.1. <i>VUs generate lumenized microvascular networks inside fibrin</i>	99
3.2. <i>Establishment and perfusion of a biomimetic fibrin-based VoC</i>	102
3.3. <i>The VUs-derived capillaries inosculate with VoC channels and form extensive microvessel networks</i>	104
3.4. <i>The VUs-derived microvascular network is perfusable</i>	105
3.5. <i>VUs promote the formation of a vascularized stroma around IOs in the VoC</i>	108
4. Discussion	110
5. Conclusions	113

SUPPORTING INFORMATION	118
Chapter IV	125
ABSTRACT	127
1. Introduction	129
2. Materials and Methods.....	130
2.1. <i>Intrahepatic Cholangiocyte Organoids (ICOs)</i>	130
2.2. <i>Image analysis</i>	133
2.3. <i>Primary cell culture</i>	134
2.4. <i>Microfluidic device</i>	134
2.5. <i>Statistical analysis</i>	136
3. Results and discussion	136
3.1 <i>Culture in FIB-LAM hydrogel supports ICOs expansion</i>	136
3.2. <i>Culture in FIB-LAM hydrogel enhances functional ICOs differentiation</i>	138
3.3. <i>Endothelial and hepatic stellate cells organize into a fibro-vascular stroma on-chip</i>	141
3.4. <i>The fibro-vascular stroma on-chip replicates a fibrogenic microenvironment</i>	143
3.5. <i>Crosstalk with reactive ICOs compromises microvessel integrity in a fibrovascular stroma</i>	144
3.6. <i>Peri-bile ductal fibrovascular stroma-on-chip uncovers secretory signals driving inflammation and fibrosis</i>	146
4. Conclusion remarks	149
Chapter V	156
Conclusion remarks and future perspectives	158

Abstract

This thesis presents the development of advanced vascularized 3D in vitro platforms designed to mimic physiologically and pathologically relevant human tissue microenvironments. Addressing well known limitations of conventional organoid systems, namely the absence of vascular and stromal support, this work introduces two complementary strategies to engineer organ-specific models, combining organoids with stromal and vascular compartments.

The first study focused on a bottom-up vascularization approach using engineered vascular units (VUs), i.e., microtissues composed of endothelial progenitor cells and organ-specific fibroblasts. When embedded in fibrin hydrogels and integrated into a custom-designed, biomimetic, vessel-on-chip (VoC) system, VUs self-assembled into perfusable microvascular networks capable of anastomosing with flow channels. In co-culture with intestinal organoids, VUs formed a supportive vascularized stroma that closely interacted with the organoids, demonstrating the feasibility and versatility of this approach for building vascularized tissues in vitro, with potential applications also in regenerative medicine approaches.

The second study established a disease-relevant model of the peri-biliary fibrovascular niche, using intrahepatic cholangiocyte organoids (ICOs) to investigate epithelial-stromal-vascular crosstalk under fibrogenic conditions. A laminin-enriched fibrin hydrogel (FIB-LAM) was optimized to support ICO expansion and functional maturation. When combined with endothelial and hepatic stellate cells in a microfluidic chip, reactive ICOs induced microvessel constriction, suppressed angiogenesis, and altered stromal cell behaviour. Proteomic profiling of the co-culture secretome revealed an enrichment in pro-inflammatory, contractile, and anti-angiogenic signals, consistent with fibrotic remodelling. This provided new insights into cholangiocyte-enhanced fibrogenesis.

Collectively, this work introduces two innovative and customizable platforms for modelling both homeostatic and disease-associated tissue environments. These models provide an important contribution to the development of physiologically relevant in vitro systems for studying organ development, fibrosis, and therapeutic response, with direct applications in tissue engineering and liver disease research.

Resumo

Esta tese apresenta o desenvolvimento de plataformas tridimensionais vascularizadas avançadas para cultura *in vitro*, concebidas para mimetizar microambientes teciduais humanos, tanto em condições fisiológicas quanto patológicas. Abordando limitações amplamente reconhecidas dos sistemas convencionais de organoides—nomeadamente a ausência de vascularização e de suporte estromal—o trabalho propõe duas estratégias complementares para a engenharia de modelos organo-específicos, combinando organoides com compartimentos vasculares e estromais.

O primeiro estudo centrou-se numa abordagem de vascularização “bottom-up” utilizando unidades vasculares (VUs), ou seja, microtecidos compostos por células progenitoras endoteliais e fibroblastos organo-específicos. Quando incorporadas em hidrogéis de fibrina e integradas num sistema biomimético “vessel-on-chip” (VoC) desenvolvido à medida, as VUs auto-organizaram-se em redes microvasculares perfusáveis, capazes de inocular com canais de fluxo. Em co-cultura com organoides intestinais, as VUs formaram um estroma vascularizado de suporte que interagiu estreitamente com os organoides, demonstrando a viabilidade e versatilidade desta abordagem para construção de tecidos vascularizados *in vitro*, com potencial aplicação também em estratégias de medicina regenerativa.

O segundo estudo estabeleceu um modelo de nicho fibrovascular peri-biliar com relevância patológica, utilizando organoides colangiocíticos intra-hepáticos (ICOs) para investigar o crosstalk entre os compartimentos epitelial, estromal e vascular sob condições fibrogênicas. Um hidrogel de fibrina enriquecido com laminina (FIB-LAM) foi otimizado para suportar a expansão e maturação funcional dos ICOs. Quando combinados com células endoteliais e estreladas hepáticas em um chip microfluídico, os ICOs reativos induziram constrição de microvasos, suprimiram a angiogênese e alteraram o comportamento das células estromais. A análise proteômica do secretoma da co-cultura revelou enriquecimento em sinais pró-inflamatórios, contráteis e anti-angiogênicos, consistentes com remodelação fibrótica. Estes resultados forneceram novas perspectivas sobre a contribuição dos colangiócitos para a fibrogênese hepática.

Em conjunto, este trabalho apresenta duas plataformas inovadoras e personalizáveis para modelar microambientes teciduais associados tanto ao estado homeostático quanto a condições de doença. Estes modelos representam um avanço significativo no desenvolvimento de sistemas *in vitro* fisiologicamente relevantes para o estudo da organogênese, fibrose e resposta terapêutica, com aplicações diretas em engenharia de tecidos e investigação de doenças hepáticas.

Abbreviations

αSMA	Alpha-smooth muscle actin
β-CAT	Beta-Catenin
2D	Two-dimensional
3D	Three-dimensional
ACTA2	alpha-smooth muscle actin gene
ANOVA	Analysis of variance
BM	Basement membrane
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
CAF	Cancer-associated Fibroblast
CCT4	Chaperonin Containing TCP1 Subunit 4
CFTR	Cystic fibrosis transmembrane conductance regulator
CK7	Cytokeratin 7
CK19	Cytokeratin 19
COL I	Collagen Type I
COL IV	Collagen Type IV
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DAPI	Diamidino-2-Phenylindole
dECM	Decellularized Extracellular Matrix
DMEM	Dulbecco's Modified Eagle Medium
E-CAD	E-Cadherin
ECFC	Endothelial colony forming cell
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EMT	Epithelial-To-Mesenchymal Transition
EDTA	Ethylenediaminetetraacetic acid
EpCAM	Epithelial cell adhesion molecule
ESC	Embryonic Stem Cell
EVS	Extracellular Vesicles
FBS	Fetal bovine serum
FIB-LAM	Laminin enriched fibrin gel
FGF	Fibroblast Growth Factor
FGF-10	Fibroblast Growth Factor-10
FN	Fibronectin
G'/G''	Elastic and viscous components of the shear moduli
GeIMA	Gelatin methacrylate
GFP	Green fluorescent protein
GO	Gene ontology

H&E	Hematoxylin and Eosin
HA	Hyaluronic Acid
HIF	Human Intestinal Fibroblasts
HGF	Hepatocyte growth factor
HSC	Hepatic Stellate Cell
HSPD1	Heat Shock Protein Family D (Hsp60) Member 1
hPSCs	Human Pluripotent Stem Cells
IGF	Insulin-like growth factor
IL	Interleukin
IGHG1	Immunoglobulin Heavy Constant Gamma 1
IGHM	Immunoglobulin Heavy Constant Mu
IGKC	Immunoglobulin Kappa Constant
iPSCs	Induced Pluripotent Stem Cells
IQGAP1	IQ Motif Containing GTPase Activating Protein 1
KRT	Keratins
LC-MS	Liquid chromatography Mass Spectrometry
Lgr5+	Leucine-rich repeat-containing G-protein coupled receptor 5
LTBP2	Latent Transforming Growth Factor Beta Binding Protein 2
MET	Mesenchymal-To-Epithelial Transition
mRNA	Messenger RNA
MMPS	Matrix Metalloproteinases
MSCs	Mesenchymal Stem Cells
MUC5AC	Mucin 5AC, Oligomeric Mucus/Gel-Forming
NO	Nitric Oxide
ON	Overnight
PBS	Phosphate buffer saline
PDGF	Platelet-derived growth factor
PEG	Polyethylene glycol
Pen/Strep	Penicillin/streptomycin
PLGA	poly (lactic-co-glycolic acid)
REG4	Regenerating Family Member 4
RPS3	Ribosomal Protein S3
qRT-PCR	Quantitative Real-Time PCR
ROCK	Rho-associated coiled-coil
ROS	Reactive Oxygen Species
RT	Room temperature
TGF-β	Transforming Growth Factor-B
VEGF	Vascular endothelial cell growth factor
VIM	Vimentin

Thesis Outline

This thesis is divided in 5 chapters with the following contents:

Chapter I presents a comprehensive literature review on the fundamental principles and recent advancements in vascularized microtissues, referred to as Vascular Units (VUs), as building blocks for bottom-up tissue engineering. The chapter discusses biofabrication strategies, the role of extrinsic and intrinsic factors on VUs formation and vascularization potential, the integration of VUs with other technologies for building complexity, their potential applications for tissue repair and in vitro modelling, and current challenges in VU-based systems.

Chapter II provides a brief overview of current strategies to promote vascularization in organoid cultures, emphasizing their importance for advancing organoid models. It also outlines the key characteristics of intrahepatic cholangiocyte organoids (ICOs), used in this thesis, and describes the vascularization strategies applied to this specific organoid type.

Chapter III introduces a novel microvascularization strategy that utilizes intestinal VUs as building blocks to generate perfusable, stromal-embedded microvascular networks in-gel. Using a custom-made, biomimetic, fibrin-based VoC platform, we demonstrated that VU-derived capillaries inosculate with capillaries from other VUs and with endothelialized microfluidic channels, establishing a perfused microvascular network throughout the bulk hydrogel on-chip. This facilitates the formation of a supportive, vascularized stroma surrounding multiple intestinal organoids (IOs) within a single device.

Chapter IV Chapter IV presents a biomimetic peribiliary vascular plexus (PBP)-on-chip platform designed to model the complex crosstalk between epithelial, stromal, and vascular compartments under fibrogenic conditions. After establishing that ICOs could expand and differentiate within a defined FIB-LAM hydrogel, the platform was used to investigate their interaction with a fibro-vascular stroma composed of ECFCs and pre-activated HSCs. This tri-culture configuration revealed a self-amplifying loop of epithelial–stromal–vascular signalling that led to fibrogenic inflammation, vascular contraction and impaired angiogenesis. Additionally, the chip microenvironment promoted the activation of ductular reaction pathways in ICOs, recapitulating hallmark features of pro-fibrotic hepatic remodelling in vitro.

Finally, **Chapter V** provides some final conclusions, with a global critical analysis of the preceding chapters, where main accomplishments are highlighted along with future perspectives.

Section A - General Introduction

Chapter I

Vascularized microtissues for bottom-up engineering of microvascular networks

lasmim Orge^{1,2,3}, Silvia A. Ferreira^{1,3} and Cristina C. Barrias^{1,2,3}

¹ i3S – Instituto de Inovação e Investigação em Saúde, Rua Alfredo Allen, 208, 4200-135 Porto,
Portugal

² INEB - Instituto de Engenharia Biomédica, Universidade do Porto, Portugal

³ ICBAS-Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Portugal

To be submitted as a review paper

1. Microvascular engineering approaches

In recent decades, the fields of tissue engineering and regenerative medicine have made significant progress. However, the development of functional vasculature remains a major challenge in creating both implantable tissue constructs and physiologically relevant in vitro models^[1-3]. A key obstacle lies in engineering the microvasculature – an intricate network of small vessels essential for oxygen and nutrient delivery, waste removal, and cellular communication, which accounts for the largest vascular area in the body.

For implantable constructs, inadequate vascularization limits the survival and integration of engineered tissues, severely restricting their therapeutic potential. Without a functional microvascular network, implanted tissues cannot sustain sufficient oxygen and nutrient exchange, leading to necrosis and compromised long-term function. In parallel, in vitro models that lack vascular structures fail to replicate the essential role of blood-perfusible networks in maintaining cellular activity and communication. This limits their ability to accurately capture the complexity of native tissue environments, thereby constraining their utility in disease modelling, drug testing, and tissue development studies.

Microvascular networks can be engineered using distinct approaches, which can broadly be classified as i) geometrically defined microchannel systems (a ‘top-down’ approach), ii) self-organized microvascular networks (a ‘bottom-up’ approach), and a iii) combination of both^[1-4]. Geometrically defined vascularization approaches involve pre-fabricating microchannels that serve as vessel templates, which can then be seeded with endothelial cells (ECs) to form hollow, perfusable vascular-like structures. Various approaches have been explored to fabricate microchannels with increasing resolution and architectural complexity, such as photolithography, spatial laser degradation, and 3D bioprinting^[1-3]. While these techniques offer precise control over vessel geometry, size, and spatial arrangement, they also present several challenges. Notably, fabricating microvessels at capillary-scale resolution remains a huge limitation due to current technological constraints^[1-3]. Also, the seeding of cells into capillary-sized structures may lead to lumen occlusion and uneven cell distribution, while adding mural and/or parenchymal cell types can be technically demanding or even unfeasible. Additionally, these approaches often overlook fundamental vascular morphogenic processes, such as angiogenic sprouting and extracellular matrix (ECM) remodelling, as well as maturation and reorganization of the neo-microvessels, limiting their physiological relevance.

Unlike pre-patterned microchannels, which impose fixed geometries on vascular structures, capillary networks in the body emerge through a highly dynamic and adaptive process. ECs self-organize in response to local biochemical and biomechanical microenvironmental cues, forming networks that continuously remodel to meet the metabolic demands of surrounding

tissues^[2, 3]. This plasticity is a defining feature of the natural vasculature, allowing vessels to grow, regress, and reorganize as needed. Self-organization driven vascularization approaches ('bottom-up') attempt to replicate these biological mechanisms and harness the intrinsic capacity of ECs to self-organize into microvascular networks, without any templating structures to guide their development^[2, 3]. Microvascularization through a bottom-up approach primarily relies on the combination of i) vasculogenesis – the *de novo* assembly of endothelial progenitor cells into capillaries, ii) angiogenesis – the growth and expansion of new capillaries from pre-existing vessel-like structures, and iii) anastomosis – the interconnection between capillary sprouts to establish a microvascular network^[2, 3]. These processes are typically promoted by co-culturing ECs with supportive stromal cells within a suitable hydrogel-based 3D matrix, which provides the necessary biochemical and biomechanical cues for robust vascular network formation. Additionally, pro-angiogenic growth factors, such as vascular endothelial growth factor (VEGF), can be added to stimulate and accelerate microvessel development, enhancing the formation of functional capillary-like networks. While replicating the full complexity of native microvascular dynamics remains a challenge, self-assembled networks ultimately create more physiologically relevant vascularization solutions than geometrically defined ones.

An alternative to embedding isolated ECs and stromal cells within a hydrogel involves the use of pre-assembled microtissues, in which these cells are first organized into small, structured multicellular aggregates before being incorporated into a 3D matrix. These pre-formed, 'vascularized' microtissues – referred to as Vascular Units (VUs) – provide a more organized starting point for capillary formation, potentially enhancing both the speed and complexity of vascular network development^[5]. Depending on their fabrication method, VUs can be broadly classified into two categories: scaffold-free and scaffold-based. Scaffold-free VUs typically take the form of spheroids, where cells self-assemble and produce their own ECM without external structural support. In contrast, scaffold-based VUs involve embedding or seeding ECs and stromal cells onto microparticles or within microgels^[6, 7] which may offer tuneable biomechanical and biochemical cues to guide microvessel organization. Because cells within VUs are already partially organized and often supported by a local ECM (either self-secreted or scaffold-associated), these strategies can potentially promote more efficient vascular self-assembly compared to approaches relying on dispersed cells only.

As discussed in this work, VUs can be used as component of in vitro 3D models or used in regenerative medicine therapies to promote functional tissue (re)vascularization, being either directly injected into the damaged tissue using a minimally invasive approach or combined into larger-scale 3D constructs prior to implantation. Different techniques can be used to assemble

VUs into more complex microvascularized engineered tissues. In recent years, advances in hydrogel design, 3D bioprinting technologies, and microfluidic devices are providing new opportunities to build complexity and biological relevance of VUs.

The following sections provide an overview of recent advances and emerging strategies for the biofabrication of scaffold-free VUs, which were the ones explored in this thesis. They cover methods for VUs formation, the influence of cellular composition and microenvironmental cues on the self-organization process, and the application of VUs in tissue engineering and in vitro disease modelling. In addition, current limitations are highlighted, and key challenges are outlined to advance the integration of VUs into more complex tissue systems.

2. Biofabrication of VUs

2.1. Methodologies for VUs formation

Scaffold-free vascularized microtissues, or VUs, are self-organized 3D microaggregates of ECs, often combined with stromal cells, designed to mimic a vascularized tissue microenvironment at the micro-scale. Methodologies for generating scaffold-free VUs leverage the intrinsic ability of these cells to self-organize into 3D structures without the need for external scaffolding^[8-10].

One of the most widely used techniques relies in the use of commercially available multi-well plates, such as V-shaped or U-shaped plates, where cell aggregation into multicellular structures, often spherical-shaped (i.e., spheroids) is encouraged by the physical confinement provided by well geometry combined with the non-adhesive properties of the substrate^{[11] [12] [13] [14]}. However, this strategy has some limitations, mainly related to scalability issues and inconsistent spheroid size and shape, which can reduce reproducibility in experimental outcomes^[15]. Similar strategies include other types of microwells, such as (micro)thermoformed^[16], agarose-casted ^[17], photo-patterned^[18] and laser-ablated ones ^[19], which also rely on physical confinement and non-adhesiveness to drive cell aggregation by gravity and proximity. The agarose-casted approach, which was the one selected for this thesis, is based on the use of micro-patterned negative moulds, which are commercially available (e.g., MicroTissues® 3D Petri Dish®)^{[17] [20] [21]} but can also be custom made. These molds serve as templates where non-adherent microwell arrays made from agarose hydrogel are cast, allowing the formation of hundreds of highly uniformly sized spheroids of different size and shape, depending on the mold's features. The molds can be designed to fit into the wells of standard tissue culture plates, enhancing high-throughput spheroid fabrication, thus making it a reliable choice for scalable applications^{[22] [23]}.

Another well-established approach for spheroid formation is the hanging drop method^{[24] [25]}, involving pipetting small volumes of a cell suspension onto a surface forming droplets (e.g., on cell culture plate lids), which is then inverted promoting gravity-driven cell aggregation and spheroid formation. Despite its technical and economic advantages due to its simplicity, the traditional hanging drop method has several limitations. These include difficulties in medium exchange without disturbing the spheroids, medium evaporation from the small droplets, and challenges related to automation and scalability^[15].

More recent biofabrication approaches include drop-on-demand techniques, such as inkjet printing or microdroplet deposition, which enable the precise dispensing of cells in controlled microdroplets onto a surface^[26, 27]. Droplet-based extrusion bioprinting^[28] and microfluidic emulsification systems^[29] have also been employed to rapidly generate large numbers of spheroids^[26]. These technologies offer greater spatial control over cell placement and can guide the formation of VUs with high precision. However, their implementation often requires specialized and costly equipment, which may limit their accessibility and scalability in certain research or clinical settings. To enhance the structural compacting potential on formation of stable VUs, cell coatings that improve cell-cell adhesion have been explored. A recent study has showed that coating cells with nanoscale biomimetic ECM and inducing spheroid formation by layer-by-layer self-assembly increased the pre-vascularization of dermal papilla VUs^[30]. Another recent study induced self-assembling by using nucleic acid nanostructures to coat cell surfaces. These DNA origami nanostructures mimic the function of cell adhesion molecules, promoting rapid spheroid formation through sequence-specific DNA hybridization. This advanced approach ensures scalability and uniformity of VUs, while enabling highly specific sequence-based interactions. These interactions allow for precise spatial arrangement of cells, facilitating the creation of customized spheroids with programmable 3D architecture, enhanced tissue-like complexity, and controlled positioning of multiple cell types to better emulate native microanatomy^[31]. These methods will be further discussed in subsequent sections.

3. Intrinsic factors affecting VUs structural and functional properties

Understanding how the integration of biological, biochemical, and biophysical factors collectively contributes to the structure and function of VUs is essential for advancing these microsystems. Intrinsic factors affecting VUs' structural and functional properties refer to the inherent characteristics of the VUs, which directly impact their ability to support the formation of primitive vascular beds or serve as building blocks for generating functional vascular networks. These factors are primarily biological and cellular in nature and include cell sources, cellular composition, and cell-cell as well as cell-matrix interactions. Investigating the interplay

between these factors is crucial for guiding the rational design of VUs that are tailored for specific application.

3.1. Cell sources

VUs are primarily composed of endothelial cells (ECs), the blood-vessel-forming cells, which can be derived from various sources. Significantly, ECs have different molecular signatures^[32], tissue-specific phenotypic plasticity and heterogeneity^[33] as recently demonstrated by single-cell RNA sequencing^[34] and reviewed in^[35] [8]. For example, depending on the tissue/organ, the endothelium can be continuous (e.g., brain), fenestrated (e.g., pancreas), or discontinuous (e.g., liver)^[36]. Furthermore, in diseased tissue, endothelium dysfunction is characterized by a distinct pathological molecular signature^[35]. A more detailed review of these cellular characteristics and their roles in vascular unit formation can be found elsewhere [37] [35].

Human umbilical vein ECs (HUVECs) are among the most frequently used cell type for VUs formation due to their affordability and reliability in long-term culture, allowing high reproducibility and comparison among studies. However, HUVECs are terminally differentiated cells that exhibit their own tissue-specific phenotype, what limits their ability to replicate the specialized responses of tissue-specific endothelial cells, making them less ideal for modelling the unique characteristics of microvascular environments^[38]. They are also not suitable for clinical application. On the other hand, mature ECs isolated from target tissues provide a more accurate representation of the vascular beds found within specific organs, but are more difficult to obtain and may quickly lose their phenotype during in vitro expansion^[39], failing to replicate the tissue-specific behaviours observed in vivo^[40].

Endothelial progenitor cells (EPCs), including late EPCs also known as endothelial colony-forming cells (ECFCs) or outgrowth endothelial cells (OEC), are blood-derived vascular populations that show robust proliferative potential and sprouting vascular ability^[41]. They can also differentiate into specific endothelial lineages^[42] and functional anastomose with host vessels upon transplantation^[43]. They represent a highly promising cell source for engineering vascularized microtissues, as they can form neovessels through both angiogenic and vasculogenic mechanisms. These have the potential to integrate with larger-caliber vascular structures, supporting perfusion in both in vitro^[44] and in vivo systems^[20].

The use of embryonic stem cells (ESCs) and pluripotent stem cells (PSCs) is challenging, and their differentiation into mature ECs continues to face technological hurdles and regulatory uncertainties^[45]. Additionally, the long-term efficacy and large-scale, reproducible manufacture of induced pluripotent stem cells (iPSCs) remains a challenge, as the protocols for differentiation and maturation are difficult to replicate and lack standardization^[46]. However,

tissue-specific ECs^[47] derived from iPSCs (iPSC-ECs) may mitigate the risk of immune rejection in autologous microtissue therapy, highlighting their potential clinical relevance^[48]. iPSC-ECs can form vascular networks in vitro^[49] and in vivo and display the molecular signature of mature vessels^{[50] [51]}. Primary cells sourced from donors can be used to build models of a pathological condition reflecting the clinical population phenotypic variability. Moreover, cell engineering is fast evolving and will offer new solutions^[52]. iPSCs allow the use of patient-derived cells to investigate genetic diseases, identify drug targets, dissect disease mechanisms and develop personalized medicines. iPSCs also offer the opportunity to create isogenic microtissues in which all cells are derived from the same donor^[11]. Moving forward, genetically engineered tissue-specific ECs will provide an additional source that can take on transcriptional properties of the surrounding tissue^[37]. Bezenah et al. studying whether iPSC-ECs could form robust, stable microvasculature as other sources of ECs like HUVECs^[49]. iPSC-ECs showed a significantly lower angiogenic sprouting capacity when compared to the HUVECs^[49]. This indicates that although there are robust published protocols for ECs differentiation from iPSCs^{[11] [53]}, the field still needs to work on further technical improvements to uniformize the functional characteristics of these cells.

Most of the VUs described in the literature also include supporting cells in their composition, such as pericytes, fibroblasts, or mesenchymal stem/stromal cells (MSC), which contribute to the stability, maturation, and functional integrity of the nascent microvessels. These supportive cells help in maintaining the proper functionality of the endothelial layer and promote the formation of a more physiologically relevant vascular structure. Among supporting cells, pericytes have key regulatory functions throughout angiogenesis, via paracrine and juxtracrine signalling, during ECM remodelling, development, maturation and stabilization of the microvascular networks. MSCs are perivascular progenitor cells^[54] that secrete immunomodulatory and pro-angiogenic factors^[55]. Furthermore, fibroblasts have tissue-specific genotypes^[56], synthesize and maintain the ECM mechanical properties, and secrete pro-angiogenic factors to improve vascular network and lumen formation^[48]. Mural progenitor cells can also be derived from iPSC to support vascular network formation^[57].

The EC and supporting cell source should ideally match the application of the VUs. Depending on the specific application, VUs can also contain parenchymal cells, which are necessary for emulate the specific functions of the tissue or organ being modelled, such as osteolineage cells (bone^{[58] [59]}), cardiomyocytes (myocardium^[60]), hepatocytes (liver^[61]), neural cells (central nervous system^[62]), or cancer cells (tumour^{[63] [64] [65]}).

3.2. Cell composition

Several studies have demonstrated that the cellular composition of VUs significantly influences their structural and functional outcomes. Key factors include i) the type of ECs, ii) the presence or absence of stromal support, iii) the specific identity of the stromal cells, the iv) EC-to-stromal cell ratio, and v) the total number of cells per VUs, which directly correlates with their size. Each of these parameters plays a critical role in modulating vascular morphogenesis.

To better understand the effects of cell composition on VUs structure and performance, high-throughput production and multiplexed analysis are invaluable tools. A recent study developed a hanging drop microarray platform capable of producing homogeneous, size-gradient, or composition-gradient spheroid arrays at high-throughput^[66]. This study confirmed that the size and shape of VUs depend on the cell ratio and culture duration, as demonstrated by several authors. In this case, composition-gradient arrays showed that the area of microvessel networks formed in fibrin hydrogels improved when the relative proportion of fibroblasts-to-ECs increased in VUs composed of HUVECs, lung fibroblasts (LF), and BT474 cancer cells. In contrast, VUs lacking LF exhibited reduced vascular network formation, as indicated by a smaller vessel area. The inclusion of LF led to a progressive increase in vascularization across compositions ranging from 64:16:20 to 8:72:20 (BT474:LF:HUVEC), underscoring the critical supportive role of fibroblasts in promoting endothelial network development^[66].

The endothelial-to-stromal cell ratio can significantly influence the structural organization and vascularization potential of VUs. Recent work from our group^[17] demonstrated that VUs composed solely of ECFCs formed loose, irregular aggregates lacking defined structure and integrity, while cohesive VUs can be obtained when combining ECFCs with an optimized proportion of MSC (Figure 1A). However, identifying the optimal cell ratio requires balancing several factors, not only to ensure VUs stability, but also to promote effective ECs organization and the development of primitive vascular networks. This optimization is highly dependent on the specific cell types used, the extent of ECM deposition, and the synergistic interactions between endothelial and stromal cells within the VUs^{[12] [22]}. As another example, VUs composed of intestinal fibroblasts and ECFCs presented improved angiogenic sprouting length in ratios with less fibroblasts (1:1, fibroblast-to-ECFC) when compared with 5:1 ratio^[21]. Another example showed that key features such as ECM remodelling and in vivo integration with host vasculature were directly influenced by the cellular composition of VUs composed of hADSCs and HUVECs. Among the tested conditions, the 3:1 ratio demonstrated the highest vasculogenic potential, ultimately leading to the formation of arteriole-scale vasculature with a longitudinal lumen and a complex vascular network following long-term in vitro culture^[67].

Several studies have consistently demonstrated that the presence of stromal cells in VUs guides EC organization. When present at an optimal ratio, stromal cells were shown to promote similar outcomes across multiple studies^{[20] [17] [68] [69] [21]}. In such conditions, ECs, which are initially randomly distributed within the spheroid, gradually reorganize into distinct vascular-like structures. These include the formation of an outer endothelial monolayer on the VU surface and/or endothelial clusters within the core. Both configurations can subsequently contribute to the development of complex vascular networks when VUs are embedded within a hydrogel.

As can be concluded from the previous examples, the effect of cellular composition is highly application-dependent; therefore, careful optimization of the different parameters is crucial to ensure that the resulting VUs meet the structural and functional requirements of their intended use.

3.3. Cell localization, cell-cell and cell-matrix interactions

The spatial localization of ECs in VUs can directly influence sprout morphology and vascular network formation, but the outcome may depend on the characteristics of each system. In a study^[68] comparing two VU configurations formed of ECFCs and MSCs – one with ECFCs uniformly distributed throughout the spheroid (mixed) and other with ECFCs seeded on the perimeter of the MSC core (shell) – the mixed spheroids demonstrated higher sprouting potential than the shell configuration. This finding highlights the importance of direct crosstalk between ECs and supporting cells for sprouting induction, suggesting that layered assembly strategies may hinder these crucial multicellular interactions. In this particular study, this conclusion was further supported by differences in cell-cell receptor NOTCH3 expression, a key regulator of pericyte functions in angiogenesis.

Yet, a study by Kim et al ^[23] demonstrated that the position of ECs in co-cultured VUs formed of human MSCs or ASCs and HUVECs modulated the vascularization process in a different way. VUs with a core-shell architecture – featuring a thick endothelial outer layer encasing a dense stromal core – demonstrated a significantly greater total network length compared to VUs with a mixed cellular distribution. However, both groups exhibited similar sprout numbers and average branch lengths, and importantly, the study did not specify the cellular composition of the sprouts or the networks being quantified. As a result, it remains unclear whether the increased network length observed in the core-shell condition is directly attributable to enhanced EC sprouting or simply reflects contributions from non-endothelial structures, making it difficult to conclude that this configuration is inherently superior for vessel formation.

Additionally, the fusion of multiple spheroids to create vascularized microtissues revealed that core-shell VUs promoted a CD31 expression pattern suggestive of early-stage vascular

network formation within the constructs after three days of culture, a pattern more prominently observed in the core-shell condition compared to the mixed distribution group^[23].

Interestingly, Carvalho et al^[17] examined the deposition of endogenous ECM, a major component of tissues and pivotal regulator of neo-vessel formation on spheroids composed of MSCs only or ECFCs and MSC at 1:1 ratio. It was observed that short-term cultured (1 day) VUs already present large amount of ECM (fibronectin and collagen), which was randomly distributed. While this pattern remained unchanged in mature (7 days) MSC spheroids, the ECFC-MSC VUs showed a peripheral enrichment of both proteins, forming an oriented layer at the basolateral side of the endothelial layer that formed at the VUs' surface. Similarly, laminin and collagen IV expression increased during maturation, remaining randomly distributed in MSC spheroids, but co-localizing with endothelial structures at the ECFC-MSC VUs' surface and core, suggesting a role in vascular stabilization (Figure 1B). These findings highlight the critical influence of spheroid cellular composition on cell-matrix crosstalk, demonstrating that the presence of ECs modulated ECM organization, leading to more structured and polarized ECM deposition, which may be related to vascular morphogenesis and maturation.

3.4. Maturation time

The maturation time (i.e., the time of culture) plays a pivotal role in modulating both the structural organization of VUs and their angiogenic potential when embedded in hydrogels. In the early stages of spheroid following cell aggregation, ECs are primarily localized at the cell surface and discrete clusters within the VU core. As maturation progresses, ECs redistribute into a thinner, more structured endothelial layer at the periphery, and organizing into primitive vascular networks^[17, 20, 44, 70] at the core, with both structures stabilized by ECM, as discussed in the previous section. Although this maturation enhances the internal vascular architecture and structural stability of VUs, it also represents the transition from an active angiogenic state to a more quiescent phenotype. Consequently, this is associated with a significant reduction in their ability to sprout when embedded in a hydrogel. Notably, across various studies, VUs consistently exhibit superior angiogenic sprouting potential at early stages – particularly around day 1 post-aggregation – when compared to their more mature counterparts, regardless of their cellular composition^[17, 20, 44, 70] (Figure 1C).

Furthermore, VUs generally exhibit a marked decline in metabolic activity from day 1 onwards, an effect particularly pronounced in constructs with higher endothelial cell (EC) densities, such as 1:1 or 1:5 EC-to-stromal cell ratios^{[44]37}. Under these conditions, increased depletion of CD31⁺ ECs from the VU has been observed during maturation^{[44]37}. Previous studies have

reported a noticeable reduction in the number of CD31⁺ cells at the VU periphery^{[44]37}, along with decreased expression of key endothelial markers including PECAM1 (CD31), CDH5 (VE-cadherin), KDR, FLT1, and the basement membrane marker LMNA^[44]. These changes likely reflect EC loss over time, potentially due to insufficient stromal support, as previously suggested^[17, 20]. Although the precise origin of the extruded cells remains unclear, their release may contribute to the observed decline in angiogenic potential of late-stage VUs.

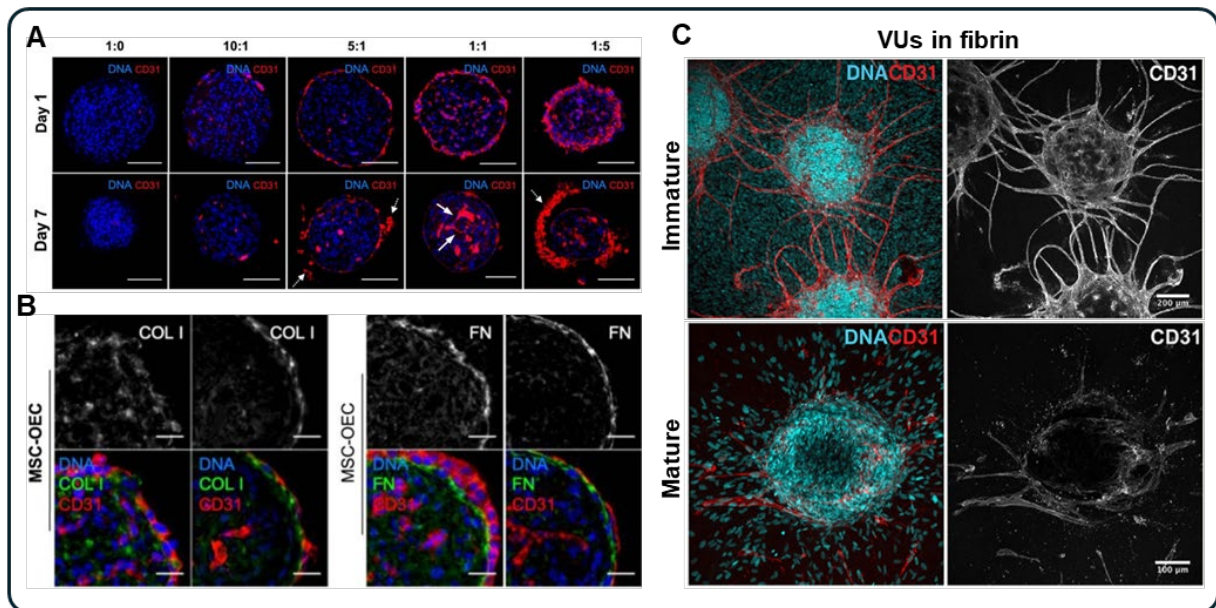


Figure 1. Effects of cell composition, ECM production and maturation time on VUs. (A) Endothelial patterning in spheroids of different MSC–OEC ratios. Scale bars:100 μ m (B) Expression of COLI and FN and co-localization with CD31⁺ cells. Scale bars:25 μ m. Adapted from Carvalho et al^[17] (Creative Commons Attribution 4.0 International License). (C) Sprouting of CD31⁺ cells from spheroids embedded in fibrin 1 day post aggregation (immature) and 7 days post aggregation (mature). Scale bars: 100 μ m. Adapted from Feijão et al^[20] (Creative Commons Attribution 4.0 International License).

4. Extrinsic factors affecting VUs structural and functional properties

Extrinsic factors also play a pivotal role in shaping the structural and functional properties of VUs. These factors arise from the surrounding environment in which VUs are cultured (or implanted) and encompass biochemical and physical/mechanical cues that influence cellular behaviour and microvessel formation. Thus, the formation, maturation, and functionality of vascular networks can be effectively modulated by modifying external conditions, such as incorporating VUs in hydrogel scaffolds, adding biochemical factors like growth factors, or applying physical forces such as shear stress.

4.1. Hydrogel matrices

The formation of complex microvascular networks from VUs is critically dependent on the presence of a hydrogel matrix, which provides a supportive three-dimensional (3D) framework that closely mimics the native ECM. Beyond structural support, hydrogels play a pivotal role in modulating cell behaviour by offering essential biochemical and biomechanical cues that influence key processes in vasculogenesis and angiogenesis, such as EC migration, alignment, sprouting, and branching. Many biocompatible hydrogels have been engineered to replicate ECM features and direct specific cellular responses, including cell adhesion, tension, remodelling, and maturation^[7]. Hydrogels with tuneable physical properties – such as stiffness, porosity, and degradation rate, can be optimized to control vascular morphogenesis and the integration of newly formed vessels. Particularly, cell-responsive and instructive hydrogels, with spatiotemporal or stimuli-dependent activity, are essential to regulate 4D cell behaviours and support critical events such as sprouting, tubulogenesis and lumenogenesis. Different hydrogels vary considerably in their capacity to support and guide vascular morphogenesis, depending on their biochemical composition, mechanical properties, and interactivity with cells^[71, 72]. The most commonly used hydrogels in VU systems include collagen, fibrin, Matrigel, dECM, GelMA, alginate, and synthetic polymers, each with distinct advantages and limitations, which are briefly discussed below.

Collagen (especially type I) is one of the most widely used natural hydrogels for vascular applications, as it is a major component of the native ECM and supports endothelial cell adhesion, migration, and proliferation. Collagen hydrogels promote endothelial sprouting and tube formation, making them highly effective for vascular network formation. However, their mechanical properties are not easily tuneable, beyond adjusting polymer concentration, and they tend to contract over time due to cell-traction and cell-mediated remodelling. This contraction can lead to changes in network stability, potentially affecting the long-term integrity of the vascular structures formed within these hydrogels^[73, 74].

Fibrin, the hydrogel explored in this thesis, is another widely used natural matrix that closely mimics wound healing environments. It provides a pro-angiogenic milieu, supporting rapid endothelial sprouting and branching. Fibrin hydrogels are FDA-approved for clinical use, notably in wound healing and tissue repair applications. However, their fast degradation rate and limited tunability in stiffness may require reinforcement or blending with other materials to improve their mechanical properties and stability for long-term culture^[17, 20, 44].

Matrigel, a basement membrane extract derived from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, is extremely potent for inducing vascular morphogenesis due to its rich

content in ECM proteins and growth factors. It robustly promotes tube formation and early vasculogenic events, making it ideal for short-term assays. However, its tumor-derived origin, undefined composition, batch variability, and lack of control over its mechanical properties make it unsuitable for mechanistic studies and limit its use in translational or long-term applications^[16, 72].

dECM-based hydrogels, derived from decellularized tissues, offer a highly bioactive and physiologically relevant matrix that closely mimics the native ECM of specific organs or tissues. These hydrogels retain the unique compositional features of the source tissue, providing a tailored environment, and may contain key bioactive molecules such as pro-angiogenic growth factors. While dECM-based materials support angiogenesis and vasculogenesis challenges such as batch-to-batch variability and limited mechanical tunability may require additional modifications for optimized use in VU systems ^[75, 76].

Gelatin methacryloyl (GelMA), is a semi-synthetic matrix derived from gelatine that can be photo-crosslinked to fine-tune its stiffness and degradation rate. It provides adequate support for vascular morphogenesis, especially when combined with pro-angiogenic signals like VEGF or co-cultured stromal cells. Its tunability and compatibility with biofabrication techniques makes it an attractive option for engineering more complex VU systems^[77, 78].

Alginate, a natural polysaccharide, is valued for its tunable properties in vascular tissue engineering. While unmodified alginate is biologically inert, it can be functionalized with cell-adhesive peptides, such as RGD, laminin-derived sequences, or other pro-angiogenic peptides, to enhance endothelial adhesion and support network formation. When modified or used in hybrid systems (e.g., combined with ECM-derived components), alginate can support vascular morphogenesis. Its primary strengths lie in its controllability, gentle cell recovery, and versatility ^[6, 7].

Synthetic hydrogels (e.g., polyethylene glycol PEG-based) offer precise control over mechanical and biochemical parameters but are biologically inert unless biofunctionalized. Like alginate, their pro-angiogenic potential is highly customizable depending on the incorporation of bioactive peptides, growth factors, or ECM-mimetic domains. When well-designed, they may offer a reproducible and versatile platform for studying and guiding vascularization^[79, 80].

Overall, natural ECM-derived hydrogels provide strong intrinsic support for vascular morphogenesis but often offer poor mechanical tunability or limited translational. On the other hand, semi-synthetic and synthetic hydrogel alginate, offer greater control and consistency, and when combined with biological cues, can eventually match the angiogenic performance of

natural matrices. To suit specific applications, additional technical considerations are also important, including the simplicity and cost-effectiveness of hydrogel fabrication, optical transparency for imaging-based analyses, and ease of cell recovery for downstream genotypic, phenotypic, and functional assessments

Although several studies have highlighted the importance of biochemical and biophysical cues in vascularization, it has generally been observed that softer, degradable, and cell-instructive hydrogels are more effective in supporting angiogenic sprouting. These hydrogels facilitate endothelial cell migration, alignment, and tube formation, which are key processes in the development of vascular networks. Additionally, their ability to degrade over time allows for the creation of more physiologically relevant tissue structures, as cells can remodel the matrix to form functional, dynamic vasculature^[72, 81].

In line with this, a recent study using VUs formed from adipose-derived stem cells (ASCs) and HUVECs demonstrated that cell proliferation, angiogenic sprouting, and ECM remodelling were enhanced within softer gelatin methacryloyl (GelMA) hydrogels (≈ 0.4 kPa) compared to stiffer hydrogels (≈ 1.4 kPa). To modulate the mechanical properties, hydrogels were submitted to 10 seconds (for soft) or 60 seconds (for stiff) of UV exposure for photo crosslinking. Under stiffer hydrogel conditions, cell sprouting from VUs was markedly limited, with no significant increases in sprout length, spheroid expansion, or cell proliferation observed beyond 96 hours. Additionally, the expression of ECM-related genes such as laminin and fibronectin, as well as the total ECM protein content, was significantly reduced in the stiff hydrogel group compared to the softer counterpart^[67]. Collectively, these findings highlight the strong dependence of cellular sprouting, proliferation, and extracellular matrix production on the mechanical properties of the surrounding microenvironment.

Hydrogel plasticity, defined as the ability to permanently deform by external traction, is another key factor. A good example of how the surrounding matrix characteristics significantly influences VUs behaviour, including cell proliferation, differentiation, and migration has been described using collagen-hyaluronic acid-based hydrogel platform with tuneable plasticity^[24]. The study analysed VUs composed of HUVECs embedded into low, medium or high plasticity hydrogels (at constant stiffness, $G' \sim 100$ Pa) and tracked for motile behaviors of ECs with protruded filopodia, known as indicator of tip cell formation and angiogenesis. Interestingly, the longest angiogenic sprouts with largest tubular lumens appeared in hydrogels with medium plasticity instead of the highest ones^[24]. This suggests that, depending on the specific conditions, highly plastic hydrogels can negatively impact vascular assembly and invasion by increasing cell contractility and downregulating VE-cadherin expression^[24].

To investigate the role of biomaterial degradability in EC:MSC-based VUs, one study^[82] employed PEG hydrogels formulated with varying ratios of matrix metalloproteinase (MMP)-degradable and non-degradable crosslinkers, creating fully degradable, partially degradable, and non-degradable environments. Constructs with higher levels of degradability supported enhanced cell spreading, sprouting, and secretion of proangiogenic factors such as VEGF and HGF, underscoring the critical role of matrix remodelling in promoting microvessel formation.

[82]

Another study utilized VUs composed of HUVECs and smooth muscle cells to systematically investigate the independent and combined effects of matrix mechanical properties, cell adhesiveness, and proteolytic degradation rate on vascular sprouting. To achieve this, the authors engineered PEG-based hydrogels with controlled variations in integrin-binding peptide (RGD) concentration (ranging from 0.7 to 3.5 mM), elastic modulus (spanning soft tissue stiffness, 0.8–4 kPa), and proteolytic degradation rate (fast vs. slow), using free-radical polymerization chemistry^[83]. The findings revealed that increasing the rate of matrix degradation significantly enhanced vascular sprout length and the number of junctions, whereas increasing hydrogel stiffness had a detrimental effect on vascular sprouting. Interestingly, variations in RGD ligand concentration alone did not significantly affect sprouting in slowly degrading hydrogels. However, under rapid degradation conditions, vascular sprout formation became highly dependent on the level of matrix adhesiveness. Lower RGD concentrations impaired endothelial sprouting, indicating that cell–matrix interactions play a critical role in permissive, remodeling-prone environments^[83].

Notably, paracrine signalling within hydrogel-embedded VUs, which mediates angiogenic sprouting, is influenced by the spatial positioning of VUs. Previous research explored the effects of different distances between VUs in fibrin gels (maintained at 400 ± 10 , 800 ± 13 , and 3000 ± 16 μm (isolated spheroids used as a control group) on vessel formation efficacy. VUs with close proximity to each other (400 and 800- μm) were seen to have higher total vessel length as compared to isolated spheroids right from the initial stages of culture. Additionally, the role of the presence of a nearby VU on the directionality of angiogenic sprouting was investigated. In accordance with previous studies on the field^[17, 20, 44], the authors observed that sprouts from neighbouring VUs were predominately oriented toward another VU, while sprouts from isolated VUs demonstrated a randomly distributed orientation. These findings indicate that the varying proximity for the diffusion of paracrine factors and cellular crosstalk may significantly affect VU communication and their sprouting capacity^[84].

Building on these findings, a recent study investigated the effects of inter-spheroid distance on VU fusion and sprouting behavior using a micropatterned, temperature-responsive hydrogel in

a sandwich culture system. VUs were positioned at defined distances of 100, 200, or 400 μm . The authors found that a 200 μm spacing provided the optimal balance – promoting directed cell migration and cellular bridge formation without triggering unwanted spheroid fusion. In contrast, a 100 μm gap led to excessive fusion between VUs, while a 400 μm distance significantly impaired sprouting, resulting in reduced cell migration and lower expression of key migration-related signaling molecules, including MAPK, MMPs, and ROCK.^[85]

4.2. Biochemical cues

Molecular signals are vital in shaping vascularization, impacting the development, function, and regeneration of blood vessels. These biochemical cues are essential in both natural biological processes and in engineered systems that mimic vascular environments like VUs. Among these players, growth factors like VEGF, FGF and PDGF are well-studied angiogenic inducers working synergistically to promote EC proliferation, migration, and tube formation^[86-88]. While many of these factors are typically present in endothelial cell culture media, additional exogenous supplementation may be employed to further promote angiogenesis and enhance vascular network formation.

In line with this, a study has reported the culture of endothelial and lymphatic VUs in a modified collagen matrix leveraging orthogonal enzymatic crosslinking with in situ tethering of a VEGF-mimetic QK peptide modified ^[89]. Both types of VUs only were able to form angiogenic capillaries in the presence of the peptides, highlighting the critical role of the tethered QK peptide – and by extension, VEGF ^[89].

Another study functionalized dextran hydrogels with RGD peptide to promote cell adhesion and an affinity peptide pair, the E/K coiled coil, to load the gels with epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF). The study demonstrated that the functionalization of the hydrogels with Ecoil-EGF and Ecoil-VEGF promotes the sprouting of VUs seeded in a supplement-depleted medium^[90].

Carvalho et al.^[17] also demonstrated VUs responsiveness to external VEGF stimulus. The addition of extra VEGF to early stage VUs (1 day post aggregation) embedded in fibrin hydrogels significantly boosted their sprouting activity, with the total sprouting length of increased to approximately the double. Significantly, both the sprouting potential and VEGF responsiveness to VEGF were abrogated in matured VUs (7 days) .

Replacing animal-derived supplements in humanized in vitro systems is crucial for developing translational strategies better suited for clinical applications. Interestingly, our group has shown that altering the cell culture media can significantly impact cell behavior and the angiogenic potential of VUs. Specifically, we demonstrated that using a pharmaceutical-grade human plasma-derived supplement as a substitute for animal-derived serum led to enhanced

outcomes. When cultured in fibrin with xeno-free media, VUs formed a more robust and organized vascularized stroma compared to those cultured with standard media containing fetal bovine serum (FBS), highlighting the importance of media composition in optimizing VUs. [20] [43].

5. Integrating VUs into advanced systems

Integration of VUs with biofabrication technologies is key for producing large-scale vascularized tissues, enabling the construction of more complex structures. It may also enhance biomimicry and facilitate the creation of perfusable channel networks and multiscale vascular architectures for sustaining tissue function. A primary challenge in this process is the ability to precisely guide the assembly of VUs into microvascular networks that extend to the whole construct. This requires anastomosis between VU-derived capillaries and/or core-level VUs fusion. These processes depend on multiple factors, including VU size, maturation state, spatial arrangement, and inter-VU distances, as well as the biochemical and biomechanical properties of the hydrogels in which VUs are embedded. This section outlines a range of strategies for promoting directed VU assembly and enhancing structural complexity, spanning from straightforward methods to advanced approaches involving 3D bioprinting and microfluidic technologies.

5.1. VUs assembly into larger constructs via direct fusion

The direct assembly of VUs can be strategically guided to create large constructs with well-defined shapes, even without complex technologies. For instance, square-shaped patch-like vascular beds were rapidly fabricated in a one-step process using over-seeded non-adhesive molds, which promoted the fusion of VUs (composed of human BM-MSCs and ECFCs) uniformly interspaced via a thin cell layer (Figure 2A) [17]. The resulting millimeter-scale tissue constructs (comprising ~256 VUs and measuring $5.33 \times 5.28 \times 0.62$ mm) exhibited a high surface area, significant endothelial sprouting potential within fibrin hydrogel, and easy scalability. In another study, tube-shaped liver-like scalable constructs were biofabricated by stacking VUs (composed of primary human hepatocytes, BM-MSCs, and HUVECs) and using a needle-array system, which empty spaces and culture with perfusion [13]. Using this strategy, the resultant millimetre-scale tissues (comprising ~500 VUs and measuring $4 \times 4 \times 5$ mm) showed improved hepatocyte function and HUVECs network formation, while those without spacing/perfusion showed cell death and tissue collapse [13].

Interestingly, while direct assembly of VUs can be promoted using VUs at different maturation stages, a recent study [22] has shown that doublet fusion using early-matured VUs (composed of myoblasts, ASCs and HUVECs) occurred more efficiently than with later-matured VUs. Still,

all maturation stages enabled VUs fusion as long as enough time is provided. This further underscores the central role of VUs maturation stage on their structural and/or functional properties, as already discussed.

The hybrid fusion of VUs with other types of microtissues forming more complex “assembloids” is also being explored. For example, Garreta et al^[76] recently, demonstrated that VUs composed of differentiated iPSCs could efficiently generate robust angiogenic sprouting networks when embedded in a collagen hydrogel, and formed 3D assembloids upon fusion with pre-vascularized kidney organoids. The assembloids developed segmented nephron-like structures, including LTL-positive proximal tubules and NEPHRIN-expressing podocyte-like cells and an extensive CD31⁺ vascular network also formed, with vessels localized in the peritubular space near the tubules and closely associated with glomeruli-like structures^[76] (Figure 2B).

VUs can also be employed to enhance the physiological relevance of organoid cultures. For example, the integration of VUs into kidney organoid systems has been shown to support nephrogenesis by improving vascularization and tissue maturation^[14]. In this approach, VUs (composed of human pluripotent stem cell-derived ECs) were embedded in a collagen type I matrix supplemented with growth factor-reduced Matrigel. These VUs exhibited enhanced expression of endothelial differentiation markers and demonstrated angiogenic sprouting with tubulogenic potential. When co-cultured with kidney organoids, the VUs fused to form vascularized 3D assembloids. Maturation of these assembloids on a soft hydrogel substrate further promoted self-organization, renal lineage differentiation, and increased vascular network density, offering a more physiologically relevant model of renal development ^[14].

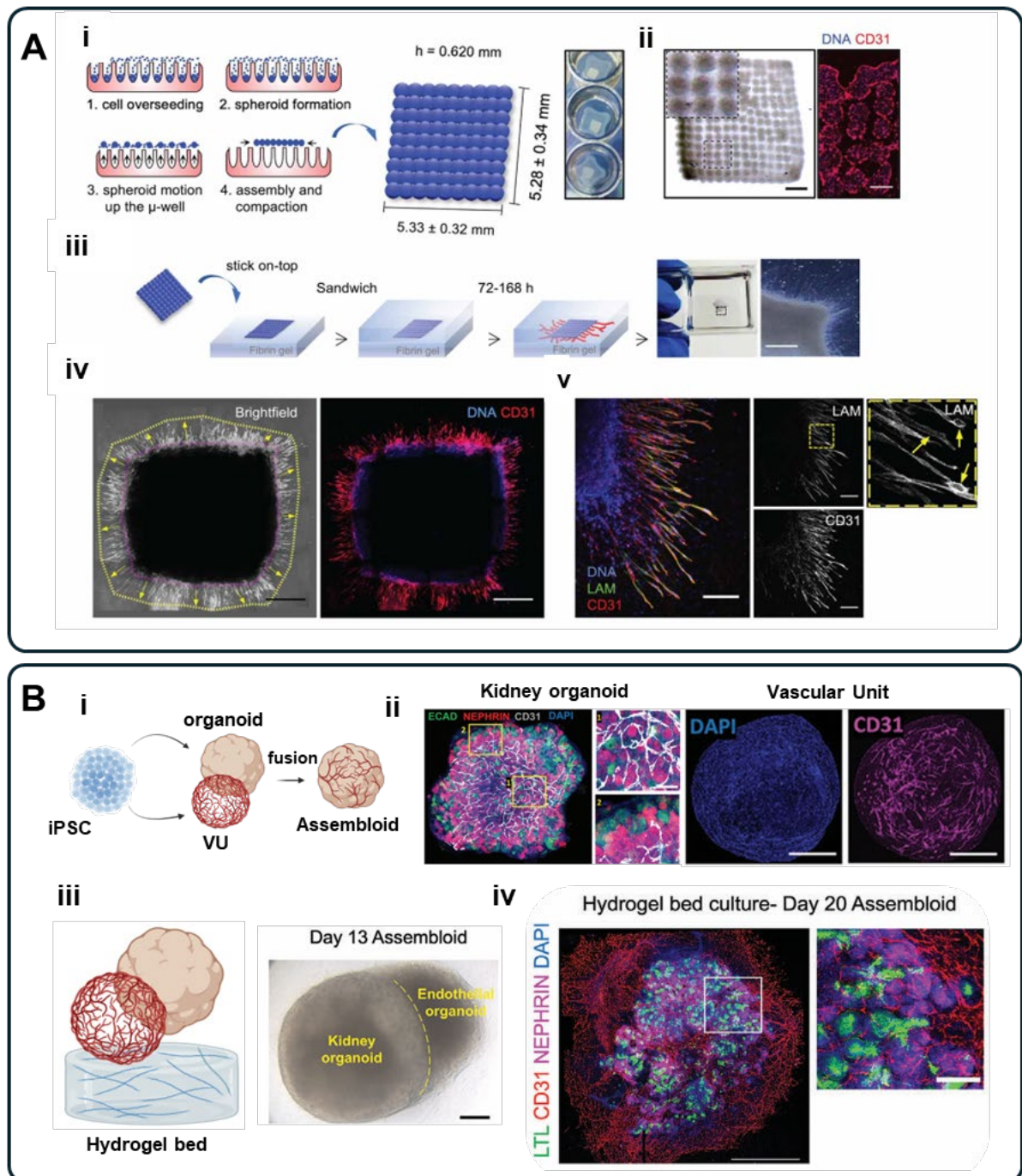


Figure 2. Overview of studies using VUs assembly into larger constructs via direct fusion. (A) (i) Schematic view of the generation of a VU-based vascularized patches. Average dimensions of each construct are illustrated. (ii) Inter-VU organization. (Scale bar: 1 mm) OEC organization (CD31) inside a 1-day-old patch. Scale bar: 250 μm . (iii) Illustration of the assay used to assess the sprouting potential of spheroid-based vascular beds. (iv) and (v) Sprouting potential upon embedding in fibrin for 168 h (iv) and 72 h (v). Scale bar: 1 mm, 250 μm (iv), (v). Adapted from Carvalho et al ^[17] (Creative Commons Attribution 4.0 International License). (B) (i) Schematics of kidney organoids assembly with VUs to induce vascularization. (ii) IF showing expression of ECAD (green), NEPHRIN (red), CD31 (grays), and DAPI (blue) in day-20 kidney organoids (Scale bars, 500, 75 μm (magnified views)), and endothelial organoids (VUs) staining for CD31 (magenta) and DAPI (blue). Scale bars, 200 μm . (iii) Scheme of the hydrogel bed culture system used for assembloid differentiation and bright field image of a day 13 assembloid. Scale bar, 200 μm . (iv) IF representative day 20 assembloid showing expression of LTL (green), CD31 (red), NEPHRIN (magenta), and DAPI nuclei staining (blue). Scale bars, 1000, 125 μm (magnified views). Adapted from Garreta et al ^[76] (Creative Commons Attribution 4.0 International License).

5.2. VUs assembly using 3D printing

VUs can be effectively integrated into biofabrication strategies as modular bioink components due to their small size, uniform geometry, and potential to promote branched vascular structures within bioprinted constructs, making them compatible with different platforms ^[91]. Recent advances in bioprinting techniques (e.g., droplet-based, extrusion, aspiration-assisted and photoablation-guided bioprinting) combined with the development of highly tuneable and bioactive bioinks have significantly enhanced the biofunctionality of VU-laden constructs. Importantly, combining VUs with advanced bioprinting techniques provides the opportunity to engineer hierarchical vascular networks by integrating microvascular beds formed by VUs with larger-caliber, bioprinted vessels. This multi-scale vascularization strategy holds great promise for the development of thick, perfusable, and physiologically relevant tissue constructs. However, several technical and biological challenges remain. Not all bioinks provide a supportive environment for VU sprouting and fusion, as already discussed in section 4.1. Suboptimal biochemical or mechanical properties of the embedding matrix can hinder endothelial outgrowth and limit the formation of continuous vascular networks. In addition, parameters such as VUs sedimentation during printing, spatial patterning, and the minimal distance required between VUs to allow vascular bridging must be carefully optimized. These considerations are essential not only for construct fidelity and reproducibility but also for enabling functional vascular connectivity. Thus, careful design of bioinks and printing protocols is essential to fully leverage the vascularization potential of VUs in 3D bioprinting applications.

The following examples illustrate how VUs have been integrated into various bioprinting strategies, highlighting key practical considerations and the resulting outcomes of their application.

A recent study by Benmeridja, L. et al. demonstrated that adipose tissue VUs (composed of human ASCs and HUVECs) were able to develop primitive vascular sprouts within their core. However, following extrusion bioprinting using GelMA as the bioink, these VUs failed to exhibit angiogenic sprouting or any signs of functional vascular organization ^[92]. In another example, VUs (composed of human ASCs, foreskin fibroblasts, and HUVECs) maintained viability and cellular outgrowth potential after extrusion-based bioprinting in GelMA. However, prolonged exposure to photo-curing during the printing process significantly impaired their growth and angiogenic potential ^[93].

A promising strategy to improve the pro-angiogenic potential of photocrosslinkable bioinks is to blend them with cell-instructive hydrogels that provide better support for endothelial sprouting and vascular network formation. Fibrin, for instance, is widely recognized for its pro-

angiogenic properties, as previously discussed, but has limited printability due to its weak rheological and mechanical characteristics. To overcome this, it is often combined with structurally robust materials such as GelMA, resulting in composite bioinks that maintain favorable printing performance while better supporting vascular morphogenesis.

For example, in a study by Fang, Y. et al.^[94], blending fibrinogen with GelMA improved printability and modulated the mechanical and physiological properties of the bioink. This allowed VUs to form interconnected vascular networks through fusion after extrusion bioprinting, while also supporting vascularization within osteogenic tissue constructs. VU-loaded GelMA supplemented with fibrinogen has been used to fabricate a bioprinted vascular bed featuring perfusable, branched vasculatures. These structures were formed using HUVEC-seeded, macroscale channels and supported initial angiogenic sprouting. VUs composed of HUVECs and fibroblasts were bioprinted in the surrounding regions of these channels. While both the printed channels and the VUs exhibited sprouting activity, the sprouts remained at an early developmental stage, limiting the formation of interconnected vascular networks and reducing the likelihood of successful anastomosis.^[95]

Still, conventional hydrogels cannot fully replicate the biochemical complexity of native ECM in terms of composition, which may limit their ability to effectively guide cellular behavior and tissue-specific organization. To address these limitations, incorporating tissue-specific dECM into bioink formulations has emerged as a promising strategy to enhance vascular network formation, while promoting more physiologically relevant tissue function^[48]. For example, an alginate-based bioink supplemented with porcine heart-derived dECM and/or collagen was shown to promote crosstalk between vascular and cardiac progenitor cells within VUs, enhancing the expression of tissue-specific markers such as cardiac troponin C and PECAM-1 ^[96].

Several other multi-component bioinks have been explored to improve vascularization and tissue organization in bioprinted constructs. For instance, extrusion bioprinting of a VU-loaded fibrin–gelatin–hyaluronan-based bioink, in combination with a layer-by-layer polycaprolactone (PCL) framework and a sacrificial gelatin hydrogel, enabled the fabrication of centimeter-scale cardiac patches featuring well-organized microvasculature. These patches, containing randomly distributed VUs, exhibited significantly higher vascular density compared to constructs printed with single-cell suspensions. Moreover, the engineered cardiac tissues displayed key structural and functional features, including lumenized vessels, aligned sarcomere architecture with intercalated discs, and synchronous contraction under mechanical stimulation ^[25]. This example underscores how increased complexity in bioink composition and

printing strategy can improve vascular and tissue outcomes. However, such approaches may also raise challenges in terms of reproducibility, scalability, and translational feasibility.

Apart from the hydrogel properties, the positioning of spheroids has also been described as an important factor for vascular formation. Ayan et al. ^[84] developed a hybrid bioprinting approach that leverages aspiration forces to selectively pick and position VUs with high spatial accuracy. Their study demonstrated that the relative proximity of VUs to each other and to perfusion channels is critical for the establishment of functional vascular networks. VUs positioned closer together (varying distances from 400 to 3000 μm) exhibited significantly greater total vessel length compared to more isolated spheroids, even at early culture stages^[84]. This enhancement is likely driven by direct cell-cell interactions and increased paracrine signalling, which together promote more robust sprouting behaviour and the formation of well-organized vascular networks (Figure 3).

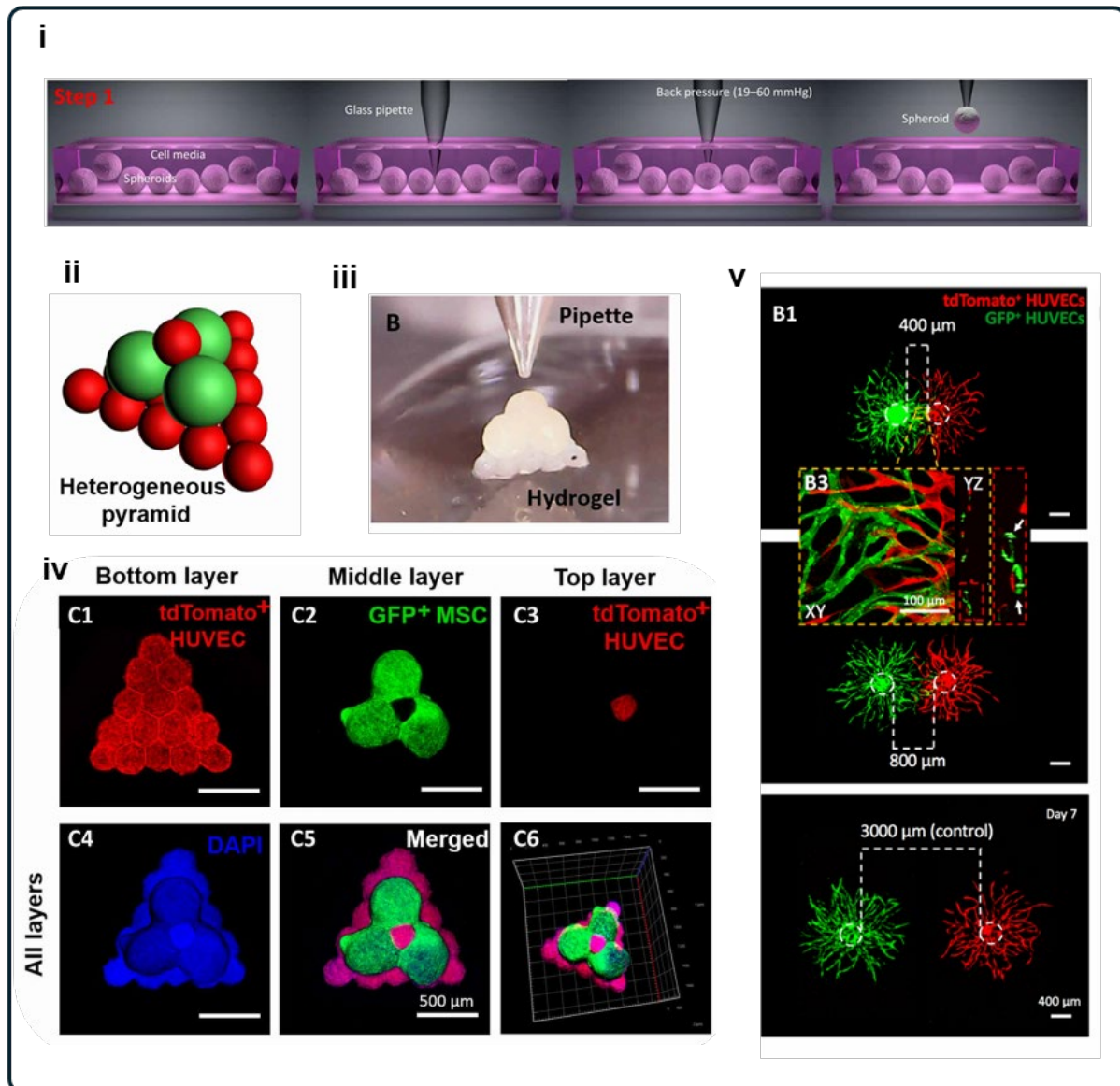


Figure 3. VUs assembly using 3D printing. (i) Step-by-step illustration of picking and bioprinting of spheroids. In step 1, spheroids are picked from the cell media by a glass pipette, where required back pressure is set to lift spheroids. Afterward, spheroids can be bioprinted into sacrificial hydrogels (scaffold-free bioprinting) or functional hydrogels (scaffold-based bio printing). (ii) A schematic illustration of 3D bioprinting of a heterogeneous pyramid construct using different sizes and types of spheroids. (iii) A photograph of the bioprinted three-layer heterogeneous pyramid. (iv) (C1 to C6) 3D reconstruction of confocal images of bioprinted pyramid of tdTomato⁺ HUVEC spheroids (first and third layers) and GFP⁺ MSC spheroids (second layer). (v) Epifluorescent images of bioprinted GFP⁺ and tdTomato⁺ HUVEC spheroids with varying distances (400 to 3000 μ m) apart on day 7 along with higher-magnification confocal images of the interface region in XY and YZ planes showing capillaries formed by both GFP⁺ and tdTomato⁺ HUVECs (indicated by white arrows). Adapted from Ayan et al ^[84] (Creative Commons Attribution 4.0 International License).

5.3. VUs integration in microfluidic platforms

The integration into microfluidic devices can significantly enhance the maturity and expand the applications of VUs by providing a perfusion source and physiological-like shear stress, which are essential for maintaining a functional endothelial phenotype. Additionally, microfluidic

devices may facilitate the study of the crosstalk between distinct stromal cells and VU-derived vascular networks ^[8]. Common designs for vascularized microfluidic devices typically combine two main strategies: the use of pre-patterned hollow channels that can be seeded with ECs to create a biomimetic vessel and the self-assembly of VU-derived ECs to form a microvascular bed. Both approaches generally involve the addition of an ECM-like hydrogel matrix within the chip's designated zone, where the embedded ECs and supporting cells can grow and organize into functional, perfused multi-scale networks ^[97].

The use of needles as microchannel templates (typical diameter: 150–550 μm), pioneered by the Tien's Lab^[98], is one of the earliest techniques for creating pre-patterned perfusable vessel-like structures. In a recent study from our group, we demonstrated the use of intestinal VUs (composed of intestinal fibroblasts and ECFCs) as building blocks to form robust, lumenized, and perfusable vascular networks interconnected with biomimetic templated vessels ^[21]. Notably, the customized device featured an open-top configuration, enabling the removal of the entire vascularized construct from the chip for downstream analyses or in vivo studies. This system also functions as a scalable in vitro platform, capable of vascularizing multiple organoids simultaneously ^[21]. The VUs facilitated the formation of vascular bridges that effectively inosculated with angiogenic sprouts from the templated vessels ^[21]. This bottom-up strategy enhances the physical scalability of the system, as the perfusion sources can be placed at greater distances from each other, thus increasing the total vascular area of the construct.

In another example, pancreatic VUs (composed of cardiac microvascular ECs and insulinoma βTC3 cells) were utilized to generate a vascular network within a microfluidic device connected to a templated perfusion channel^[99]. While the VUs exhibited functional responsiveness to an anti-cancer drug, thus demonstrating the model's potential for drug screening, there was limited evidence of fully formed, lumenized, and perfusable angiogenic sprouts, which are essential for validating the formation of physiologically relevant vasculature. This highlights the need for further optimization of both the cellular composition and microenvironmental cues to support robust vascular morphogenesis on-chip ^[99].

Despite these advances, the precise positioning of multiple microtissues within microfluidic devices remains a significant challenge in the field. To address this, a recent study introduced a serpentine-shaped microfluidic chip designed to enable the controlled entrapment and spatial alignment of organoids and other 3D vascularized cellular constructs such as VUs. This configuration facilitated close contact and successful inosculature of VUs with the endothelium-lined perfusion channels, enhancing vascular integration and spatial reproducibility within the device ^[100] (Figure 4C).

The importance of applying physiological levels of flow and its effect on vascular development has also been highlighted. Using the AIM Biotech® chip, a recent study developed a vascularized and perfusable cardiac model by integrating pre-vascularized cardiac VUs with an external vascular network formed through the self-organization of hiPSC-derived endothelial cells (hiPSC-ECs) and human brain vascular pericytes (HBVPs) within a fibrin-based microfluidic organ-on-chip system. The incorporation of VUs did not impair the formation of the surrounding vascular network, which developed robustly both within and around the VUs. To investigate the mechanisms underlying intra-microvascular network formation, VUs were pre-vascularized with GFP-expressing hiPSC-ECs and co-cultured on-chip with mCherry-expressing hiPSC-ECs and HBVPs. Bidirectional anastomosis between the internal vasculature of the VUs and the surrounding external vascular network was observed resulting in the formation of hybrid vessels composed of ECs from both internal and external sources. When accessing the effects of intermittent or continuous flow on the system, it was observed that continuous flow enhanced substantially the formation of hybrid vessels increasing vessel density and improving EC-cardiomyocyte crosstalk via paracrine factors, such as nitric oxide^[101].

In a similar work, functionally beating cardiac VUs were successfully vascularized within a perfusable network on-chip ^[102]. Simultaneous embedding VUs alongside ECs and pericytes during on-chip vascular bed formation led to more integrated vascularization compared to sequential seeding of VUs onto a pre-formed network. To investigate how cellular composition influences connectivity between the vascular bed and cardiac VUs, microvascular networks were formed using either HUVEC monocultures or HUVEC/pericyte co-cultures. Contrary to previous studies reporting the supportive role of pericytes in vascular formation, co-cultures resulted in less dense and less interconnected networks than HUVECs alone. The study also compared the structural and functional features of cardiac VUs cultured on-chip with those maintained in suspension or embedded in fibrin gels. In all conditions, striated patterns of F-actin and α -actinin were evident, indicating sarcomere formation. Extended culture up to 25 days yielded structurally stable VUs that retained marker expression and contractile features, confirming the maturation of cardiomyocyte-like VUs within the microfluidic system (Figure 4D) ^[102].

Another study developed a vascularized tumour-on-chip model by introducing a HUVEC suspension containing either HepG2-only spheroids or tumour–EC hybrid VUs. Following the formation of a vascular bed on-chip, a significantly greater volume of blood vessels was observed surrounding the tumour VUs compared to those with HepG2-only spheroids, highlighting the pro-angiogenic effect of incorporating ECs into tumour 3D constructs^[103].

Incorporating lymphatic vessels into organ-on-chip models is essential for recapitulating the full complexity of native vascular networks. To address this, the same study developed a platform in which blood and lymphatic vessels were simultaneously patterned around tumor VUs by introducing lymphatic endothelial cells into the perfusable vascular bed. Upon applying a hydrostatic pressure gradient (achieved by varying medium volumes at each end of the media channel) the authors observed that both vascular and lymphatic networks established direct physical contact with the tumor VU. This dynamic perfusion setup promoted the formation of a more intricate and functional vascular architecture, significantly enhancing both lymphangiogenesis and angiogenesis compared to static culture conditions^[103].

The integration of photoablation-guided bioprinting and microfluidics has emerged as a promising strategy to improve vascular network functionality and assess the network-forming potential of VUs compared to single-cell suspensions. In one study, two-photon polymerization (2PP) high-resolution bioprinting was employed to fabricate capillary-sized, branched microvascular structures (10–30 μm in diameter) directly on-chip. These structures were created using a photosensitive thiol-ene photo-click hydrogel composed of thiolated gelatin (Gel-SH) and gelatin-norbornene (Gel-NB). The printed microchannels were positioned in close proximity to embedded VUs and stromal cells within the hydrogel. Compared to single-cell suspensions, VUs demonstrated superior efficiency as modular building blocks for generating guided vascular structures, highlighting their enhanced self-organization and network integration capabilities^[104].

Building on the concept of open-top systems, a commercially available microfluidic platform (IFlowPlate™) has further advanced the scalability of vascularized tissue engineering by enabling the simultaneous generation of up to 128 perfusable vascular networks^[105] (Figure 4A). This format allows for the production of extractable, perfusable constructs and opens new possibilities for high-throughput applications, such as drug screening. In a related example, Bonanini et al. employed the OrganoPlate®, a platform containing 64 individual microfluidic chips, to investigate vascularization of hepatic tissues. In this system, hepatic VUs (composed of hepatocytes and HUVECs) were layered on top of a pre-formed, self-assembled vascular bed. Perfusable endothelial sprouts successfully infiltrated and vascularized the hepatic microtissues, and the platform was also used to model veno-occlusive disease following exposure to azathioprine^[74] (Figure 4B).

Importantly, while commercial systems offer significant advantages in terms of scalability, standardization, and throughput, they may impose design constraints that limit the biological complexity or customization possible with more tailored, in-house platforms. As such, selecting between custom-built and commercially available microfluidic systems requires balancing

factors like experimental flexibility, control over microenvironmental parameters, and the degree of physiological relevance needed, depending on the specific goals of the study.

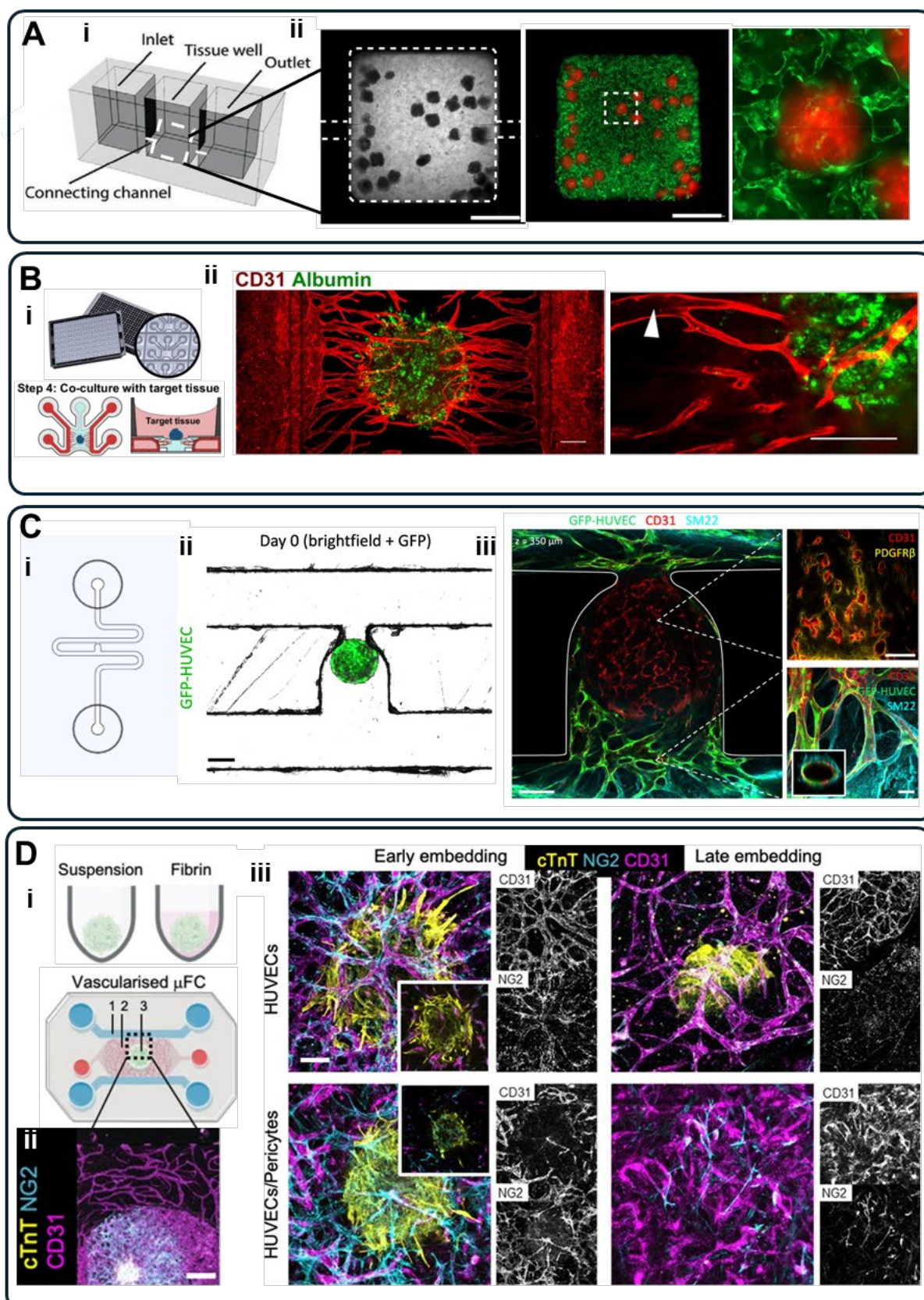


Figure 4. Overview of studies integrating VUs into microfluidics devices. (A) (i) Illustration of IFlowPlate design. (ii) Vascularized hepatic spheroids in IFlowPlate. Blood vessels are shown in green while hepatic spheroids are shown in red. White dotted lines outline the edge of the well. Scale bar, 3 mm. Adapted from Lin et al^[105] (Copyright © 2021, American Chemical Society). (B) (i) OrganoPlate Graft with 64 microfluidic units positioned underneath a 384 micro titer plate and graphical abstract. (ii) Hepatocyte spheroid co-cultured with microvessels for 7 days and stained against albumin (green) and CD31 (red). Scale bar: 200 μm . Adapted from Bonanini et al^[74] (Creative Commons Attribution 4.0 International License). (C) (i) Top and 3D view of the microfluidic device with the microchannel featuring a trapping site. (ii) Spheroid trapped on chip at day 0. (iii) Hierarchical structure of vascularized spheroid-on-chip. Adapted from Quintard et al^[100] (Creative Commons Attribution 4.0 International License). (D) spheroids were cultured in ULA plates (suspension), in ULA plate with fibrin (fibrin) and in a microfluidic device (vascularised μFC). (ii) Confocal images show vascularised CMEF spheroids after 10 days of culture in the device. Scale bar is 500 μm . (iii) Vascularised spheroids embedded at early or late time points in the devices and vasculatures formed of HUVECs or HUVECs/pericytes. Scale bar is 100 μm . Adapted from Cio et al^[102] (Creative Commons Attribution 4.0 International License).

6. VUs as advanced tools for drug testing

VUs can be used either as standalone in vitro models of disease and injury or as vascularizing components within engineered tissues and organoid cultures. In both contexts, they contribute to enhancing the physiological relevance of the system, making them highly valuable tools for drug screening, toxicity testing, and therapeutic development.

A recent in vitro model of cardiac injury and fibrosis was developed by inducing cryoinjury in VUs (composed of iPSCs-derived cardiomyocytes and ECs)^[106]. The model was treated with Captopril, a drug commonly used for heart failure therapy, which significantly attenuated cardiac cryoinjury-induced fibrosis and functional disorder^[106]. Additionally, these VUs can be used as an in vitro model for drug screening of cardiac toxicity and doxorubicin showed dose-dependent toxicity^[106].

In another example^[31], liver VUs (composed of hepatocytes, hepatic stellate cells, Kupffer cells and sinusoidal ECs) were combined with immune cells, via self-assembling using linking nucleic acid nanostructures that decorated living cells. These VUs were subjected to acetaminophen-induced injury and the impact of immune-stromal interactions was evaluated. The acetaminophen toxic concentration was higher in VUs with macrophages, which are able to efficiently metabolize and clear the drug/toxin, thereby reducing liver damage^[31]. Additionally, non-alcoholic steatohepatitis, a complex chronic disease was modelled in vitro using fibrotic VUs, which were fabricated using hepatic stellate cells pre-activated with TGF- β , to test GFT505 drug effect on fibrosis^[31]. The authors showed a standardized and straightforward platform built with fibrotic VUs (using ultra-low attachment 384-well plates) and integrated with AI for high-throughput drug screening (37 drugs tested) and high-content imaging that promises to improve drug screening efficiency for large-scale drug discovery^[31].

VUs can also be employed to model tumour responses in vitro. Tumour VUs – composed of tumour cells, ECs, and in some cases stromal cells; enable the formation of vascularized tumour-like microtissues that more accurately recapitulate cellular interactions and the architecture of the surrounding tumour microenvironment (TME). This allows for more physiologically relevant assessment of drug responses, particularly for therapies targeting tumour vasculature and the TME.

For example, tumour VU-laden hydrogels integrated into microfluidic platforms have been used as drug efficacy testing systems^{[103] [107]}. In one study, treatment of hepatic tumour-based VUs (composed of HepG2 cells and HUVECs) with Axitinib, an anti-tumour and anti-angiogenic drug, resulted in a significant reduction in blood vessel volume and decreased interconnectivity within the vascular network^[103]. In another study, Paclitaxel, a chemotherapeutic agent that induces mitotic arrest and apoptosis, was applied to breast tumour-based VUs (composed of MCF-7 cells, lung fibroblasts, and HUVECs) under continuous flow conditions. Interestingly, tumour constructs exposed to flow developed greater drug resistance – as evidenced by larger tumour size, compared to static, avascular controls^[107].

To evaluate the impact of the stromal components, tumour VUs (composed of tumour cells, dermal fibroblasts, THP-1 monocytes, and HUVECs) were generated using different tumour cell lines to recreate distinct TMEs^[69]. This was achieved by genetic manipulation targeting specific TME components (e.g., overexpression of growth factors (PDGF-B and VEGF), or transcription factors regulators of epithelial-mesenchymal transition (Snail1 and Twist1)^[69]. A VU-based array platform was employed to evaluate and rank the effects of various TME-targeted drugs with high inter- and intra-assay reliability and reproducibility^[69]. Antifibrotic and antiangiogenic drug responses were assessed using specific immunostaining, light-sheet fluorescence microscopy, and translated into multi-parametric datasets via an advanced image analysis pipeline^[69]. The entire workflow – from biofabrication, drug treatment, and sample processing to imaging and analysis; was compatible with existing liquid-handling systems, enabling automation and scalability^[69]. However, the platform has not supported high-throughput screening of large compound libraries. Other drug screening platforms used to test multiple drug combinations and doses for tumor drug resistance, by measuring their effects on tumor spheroid viability, could be adapted for future studies using VUs.^[66]

7. VUs for tissue repair in trauma and disease

There is a growing number of studies demonstrating the application of VUs in vivo for tissue regeneration following trauma or disease. This section highlights selected preclinical examples in which VUs were transplanted into different tissue environments to promote vascular integration and functional repair. Only studies involving in vivo transplantation are discussed, with particular attention given to key factors influencing regenerative outcomes. The examples were chosen to reflect applications more directly related to the cardiovascular system and to the one of the main thematic focus of this thesis, i.e., hepatic tissue. No studies related to intestinal implantation were found.

Cardiac and vascular tissue

VUs transplantation has been shown to improve the ischemic tissue recovery in an animal model of ischemic stroke^[108]. Intracranial injection of VUs (composed of UCB-MSCs and HUVECs; 100 VUs/site) following ischemic brain injury in a mouse model of permanent distal middle cerebral artery occlusion (MCAO) showed early cell engraftment and improved therapeutic efficacy in comparison to saline and single cell suspension (5,000,000 cells/site)^[108]. Moreover, VUs transplantation showed neuroprotective, pro-neurogenic, pro-angiogenic and anti-scarring potential^[108]. Indeed, VUs transplantation ameliorated poststroke glial scarring and promoted neurogenesis, angiogenesis, induced formation of clear ECM structure and increased structural and motor brain functional recovery^[108]. The authors highlighted that the organization into VUs resulted in a more efficient therapy compared with single cell suspension because this strategy increased in physical volume and cell survival/retention at the site of injection, improved in cell-matrix interactions during cell harvesting/transplantation, protected engrafted cells from the hostile environment in ischemic brain tissue, enhanced intercellular interactions prior and post-transplantation, increased accumulation and secretion of multiple neurotrophic, anti-apoptotic, and pro-angiogenic factors by engrafted cells.

Furthermore, VUs transplantation has shown relevant improvements in myocardial infarction^{[109] [25]}. Injection of VUs (formed of human UCB-MSC and HUVECs, 200 VUs/site) in the peri-infarct zones of Lewis rats with acute myocardial infarction (immunosuppressed with cyclosporine A) improved angiogenesis, blood perfusion recovery, ventricular contractility and cardiac function in comparison to saline and single cell suspension (1,000,000 cells/site)^[109]. In other study, VUs injection (formed of human ESCs-ECs and ESCs-derived or neonatal rat cardiomyocytes; 100 VUs/site) seemed to be more effective than single cells (1,000,000

cells/site) on fibrosis reduction and cardiac function recovery in nude mouse myocardial infarction model^[25].

Additionally, few in vivo studies have shown that VUs transplantation can improve the ischemic skeletal muscle recovery^{[110] [111] [112]}. VUs injection (formed of human UCB-MSCs and HUVECs; 800 VUs/site) in a Balb/C mouse model of hindlimb ischemia (immunosuppressed with cyclosporine A) enhanced functional vessel formation and induced blood perfusion recovery more effectively than single cells (4,000,000 cells/site)^[110]. In other study from the same group with the same mouse model, VUs were injected (100 VUs/site) in combination with N-acetylcysteine (NAC), an antioxidant ROS scavenger. The NAC-mediated reduction of oxidative stress following VUs transplantation improved cell adhesion, retention, and survival in the ischemic tissue, promoting higher therapeutic angiogenesis that led to significant blood flow recovery and limb salvage in comparison to saline and monotherapy^[111].

Another group looked at the benefits of combining VUs (composed of MSC and HUVECs) with nanoceria-decorated graphene oxide (CeGO), known for its strong reactive oxygen species (ROS) scavenging properties and its ability to provide cell-matrix interaction signals^[112]. When VUs were exposed to oxidative stress induced by H₂O₂ in vitro, those cultured with the antioxidative CeGO material exhibited enhanced survival rates, improved ROS scavenging, and demonstrated both anti-apoptotic and pro-angiogenic functions^[112]. Injection of CeGO-VUs (389 VUs/site) in the nude mouse ischemic hindlimb enhanced blood reperfusion, tissue recovery, and limb salvage (~60% CeGO-VUs vs. ~29% in VUs vs. 0% in PBS control).

Hepatic tissue

The same hepatic VU-based construct previously described in Section 5.1 was also evaluated for in vivo application. This human liver-like tissue assembled from approximately 200 VUs (composed of BM-MSCs, hepatocytes, and HUVECs), demonstrated self-organization and successful engraftment onto the transected liver parenchyma of nude rats. Although a vascular network formed in vivo, hepatic function remained limited, likely due to insufficient blood perfusion and progressive fibrosis. These findings underscore the importance of achieving a higher degree of vascular maturation and functional pre-conditioning in vitro prior to transplantation^[13].

Bioprinted hepatic tissue constructs with VUs (composed of human foreskin fibroblasts and HUVECs with or without HepG2 hepatic cells) in a GelMA-fibrinogen bionk showed neovascularization at day 7 post-implantation subperitoneally in mice^[95]. To support the survival of the multiscale vascular network under physiological blood flow, the construct included an inner and external pressure-bearing printed layer to enable the direct surgical

anastomosis of the carotid artery to the jugular vein of adult Sprague-Dawley rats^[95]. The implanted hepatic construct survived for 4 days until cracking due to the high blood pressure in vivo. One week after the implantation, thrombus was observed, and the tissue was removed. VUs can be used either as standalone in vitro models of disease and injury or as vascularizing components within engineered tissues and organoid cultures. In both contexts, they contribute to enhancing the physiological relevance of the system, making them highly valuable tools for drug screening, toxicity testing, and therapeutic development.

8. Challenges, industrial outlook and Clinical translation

Until now, only few engineered vascularized tissues for surgical repair of macrovessels have been translated into the clinic successfully^[113]. For the treatment of ischemic diseases at the microvascular level, most therapeutic strategies in clinical trials involve growth factors or gene delivery. Cell therapies are currently being tested under clinical trials (e.g., autologous EPCs for treating acute myocardial infarction (NCT00936819) or liver cirrhosis (NCT03109236); MSCs for treating lung injury (NCT04447833). Only a few clinical trials are testing microtissues (e.g., lung stem cell spheroids transplant in patients with idiopathic pulmonary fibrosis (NCT04262167); iPSC-derived cardiomyocyte spheroids transplant in patients with heart failure (NCT04945018); and iPSC-derived pancreatic islets transplant in diabetic patients (NCT04786262). Considering evidence in vitro showing that VUs (composed of MSC and HUVECs) loaded in collagen/fibrin can be more efficient than MSC-only spheroids in osteogenic differentiation and pre-vascular network formation, it can be expected that following further studies, VUs clinical translation will represent major shift in cell therapy and tissue repair^[114].

Compared to the current therapies that rely on the exogenous administration of free cells, VUs injection promises to offer the advantage to repair ischemic tissue in a spatiotemporally controlled manner^[113]. Preclinical studies have shown that VUs can improve anastomosis, reperfusion and host-microtissue integration conducive to tissue repair in trauma and disease^[37]. However, before translation to the clinics, further comprehensive studies focusing on vascular morphogenesis and inosculation with the pre-existing host's vasculature are still required to ensure perfusion and functional integration. VUs transplantation must be proved biosafe, preclude severe immune reactions, rejection, and tumorigenicity. Of note, most reported in vivo studies used immunodeficient animals or immunosuppression schemes disregarding the valuable contribution of the immune system to neovascularization and tissue repair.

To reach the market, the engineered VUs should be designed with a reproducible, time, and cost-effective fabrication approach to be scalable, high throughput, automated, physiologically relevant and simple to manipulate^[8]. The challenge of selecting appropriate cell sources, along with the extensive reliance on cell lines and insufficient reproducibility have contributed to hindering the clinical translation of VUs. The lack of standardization in the culture microenvironment used during VUs production and maturation further complicates this issue. Advances in rejection-free ‘universal’ donor iPSCs promise to be a paradigm-shifting approach for engineering cell-based therapeutics, including VUs^[115].

For the industrial and clinical scale-up of VUs, cell large-scale expansion in bioreactor systems complying with regulatory guidelines (FDA, EMEA) and Good Manufacturing Practices (GMP) is also essential^{[116] [117] [118]}.

Nonetheless, VUs have been valuable in developmental biology^[45], modelling human health and disease conditions^{[119] [120] [63]}, and in drug screening, monitoring of therapeutic regimes and optimization of personalized and precision medicine^{[37] [63] [61] [119]}. For example, VU-based disease models convey a useful platform to validate drug efficacy and off-target effects, and provide insight into the pathological onset, progression and resolution of the disease, to identify therapeutic targets. This contributes to reducing/replacing the use of animal models in compliance with the 3R’s principle, accelerating cost-effective and life-changing solutions to patients. Additionally, VUs prepared with patient-specific cells will facilitate the investigation of rare diseases, cancer metastasis or atherosclerosis^[37]. VUs can be included in organs-on-a-chip models^{[121] [122] [46] [123] [124]}, and eventually improve the vascular-linked multi-organ-on-a-chip platforms^[125], which recreate tissue-tissue crosstalk essential for the progress on many therapeutic solutions. Biobanking and research sharing of VUs will certainly speed up the process. This field has led and will speed up multiple collaborations among academics, clinicians, start-ups and pharmaceutical industries (e.g., InSphero, Roche and Fluofarma use spheroids and Organovo or Aspect Biosystems use bioprinted models in drug development).

9. Conclusions and future outlook

Despite significant advances in recent years, replicating the inherent complexity of vascular structures using VU-based bottom-up approaches remains challenging. Of note, while broadly classified as VUs, the term is sometimes applied in the literature to describe microtissues containing ECs cells without clear evidence of organization or potential for vascular networks formation. As such, care should be taken when interpreting or comparing different systems, and criteria for defining true vascular organization should consider not only the presence of ECs, but also their spatial arrangement, network morphology, and degree of functional

maturation. Similar considerations apply to the “quality” of VU-derived vascular networks, which may show limited perfusability, spatial-temporal control, and immature hemodynamic response.

Several fundamental gaps need to be addressed to increase VUs biomimicry and physiological relevance of VU-derived microvessels. This includes a deeper understanding of basic vascular biology at hierarchical scale, and of the unique requirements of tissue/organ specific-microvasculature. Furthermore, the development of strategies to enhance VUs maturation/function will be key for the progress of this field. Bottom-up approaches will gain with the incorporation of physiological fluid flow conditions^[21], immune components^[40] ^[126] ^[127] ^[128] ^[129], lymphatic system^[130] ^[89], innervation^[131], and eventually programming factors^[132] for dynamically controlling and directing tissue generation and maturation^[9] ^[133].

In a more technological perspective, the field is developing towards more automated, controlled and integrated biofabrication processes to increase VUs uniformity and reproducibility, facilitating scale-up^[9]. This is fuelled by advances in biofabrication technologies, which can be combined, for example, with sophisticated remote assembling methods of VUs (e.g., acoustic, magnetic or optical)^[9]. Altogether, these will allow increasing complexity and production-throughput. Moving forward, advances in automation, sensing and monitoring will allow real-time and non-invasive high-throughput readouts by using embedded-sensor technologies, 3D live imaging systems, or actuators (to rapidly apply specific stimuli)^[134] providing valuable insights into the functional tissue dynamics^[40].

Computational modelling and machine learning techniques have the potential to significantly enhance biofabrication processes and the quality of engineered VUs. These approaches can accelerate formulation optimization, automate image-based quality control, and enable high-throughput analysis of multi-omics datasets (e.g., genomics, epigenomics, transcriptomics, and metabolomics) to guide VU design^[135] and validate their resemblance to native tissues.

Another emerging trend in the field is to go beyond ‘static’ setups and incorporate dynamic systems that better mimic the spatiotemporal signaling dynamics of native tissues – for example, using 4D bioprinting with smart materials that respond to external stimuli.

This multidisciplinary field depends on ongoing progress in vascular cell biology, biomaterials, and biofabrication. Recent advances, such as gene editing^[52], patient-specific cell sources, designer and/or human-derived hydrogels, and integrative biofabrication technologies^[65], are accelerating development. Growth in cross-sector collaborations, improved ethical and regulatory frameworks, and breakthroughs in artificial intelligence, including machine learning and in silico modeling^[136], are also contributing. Together, these trends are expected to drive

the development of biomimetic and functional VUs toward clinical applications in the near future.

References

- O'Connor, C.; Brady, E.; Zheng, Y.; Moore, E.; Stevens, K. R., Engineering the multiscale complexity of vascular networks. *Nat Rev Mater* **2022**, 7 (9), 702-716.
- Song, H. H. G.; Rumma, R. T.; Ozaki, C. K.; Edelman, E. R.; Chen, C. S., Vascular Tissue Engineering: Progress, Challenges, and Clinical Promise. *Cell Stem Cell* **2018**, 22 (3), 340-354.
- Murphy, A. R.; Allenby, M. C., In vitro microvascular engineering approaches and strategies for interstitial tissue integration. *Acta Biomaterialia* **2023**, 171, 114-130.
- Gutterman, D. D.; Chabowski, D. S.; Kadlec, A. O.; Durand, M. J.; Freed, J. K.; Ait-Aissa, K.; Beyer, A. M., The Human Microcirculation. *Circulation Research* **2016**, 118 (1), 157-172.
- Rahimnejad, M.; Nasrollahi Boroujeni, N.; Jahangiri, S.; Rabiee, N.; Rabiee, M.; Makvandi, P.; Akhavan, O.; Varma, R. S., Prevascularized Micro-/Nano-Sized Spheroid/Bead Aggregates for Vascular Tissue Engineering. *Nano-Micro Letters* **2021**, 13 (1), 182.
- Torres, A. L.; Bidarra, S. J.; Pinto, M. T.; Aguiar, P. C.; Silva, E. A.; Barrias, C. C., Guiding morphogenesis in cell-instructive microgels for therapeutic angiogenesis. *Biomaterials* **2018**, 154, 34-47.
- Torres, A. L.; Bidarra, S. J.; Vasconcelos, D. P.; Barbosa, J. N.; Silva, E. A.; Nascimento, D. S.; Barrias, C. C., Microvascular engineering: Dynamic changes in microgel-entrapped vascular cells correlates with higher vasculogenic/angiogenic potential. *Biomaterials* **2020**, 228, 119554.
- Dellaquila, A.; Le Bao, C.; Letourneur, D.; Simon-Yarza, T., In Vitro Strategies to Vascularize 3D Physiologically Relevant Models. *Advanced Science* **2021**, 8 (19), 2100798.
- Ouyang, L.; Armstrong, J. P. K.; Salmeron-Sanchez, M.; Stevens, M. M., Assembling Living Building Blocks to Engineer Complex Tissues. *Advanced Functional Materials* **2020**, 30 (26), 1909009.
- Gaspar, V. M.; Lavrador, P.; Borges, J.; Oliveira, M. B.; Mano, J. F., Advanced Bottom-Up Engineering of Living Architectures. *Advanced Materials* **2020**, 32 (6), 1903975.
- Campostrini, G.; Meraviglia, V.; Giacomelli, E.; van Helden, R. W. J.; Yiangou, L.; Davis, R. P.; Bellin, M.; Orlova, V. V.; Mummery, C. L., Generation, functional analysis and applications of isogenic three-dimensional self-aggregating cardiac microtissues from human pluripotent stem cells. *Nature Protocols* **2021**, 16 (4), 2213-2256.
- Giacomelli, E.; Bellin, M.; Sala, L.; van Meer, B. J.; Tertoolen, L. G. J.; Orlova, V. V.; Mummery, C. L., Three-dimensional cardiac microtissues composed of cardiomyocytes and endothelial cells co-differentiated from human pluripotent stem cells. *Development* **2017**, 144 (6), 1008-1017.
- Yanagi, Y.; Nakayama, K.; Taguchi, T.; Enosawa, S.; Tamura, T.; Yoshimaru, K.; Matsuura, T.; Hayashida, M.; Kohashi, K.; Oda, Y.; Yamaza, T.; Kobayashi, E., In vivo and ex vivo methods of growing a liver bud through tissue connection. *Scientific Reports* **2017**, 7 (1), 14085.
- Garreta, E.; Moya-Rull, D.; Marco, A.; Amato, G.; Ullate-Agote, A.; Tarantino, C.; Gallo, M.; Esporrín-Ubieto, D.; Centeno, A.; Vilas-Zornoza, A.; Mestre, R.; Kalil, M.; Gorroñogoitia, I.; Zaldua, A. M.; Sanchez, S.; Izquierdo Reyes, L.; Fernández-Santos, M. E.; Prosper, F.; Montserrat, N., Natural Hydrogels Support Kidney Organoid Generation and Promote In Vitro Angiogenesis. *Advanced Materials* **n/a** (n/a), 2400306.
- Kim, W.; Gwon, Y.; Park, S.; Kim, H.; Kim, J., Therapeutic strategies of three-dimensional stem cell spheroids and organoids for tissue repair and regeneration. *Bioactive Materials* **2023**, 19, 50-74.
- Fois, M. G.; Tahmasebi Birgani, Z. N.; López-Iglesias, C.; Knoops, K.; van Blitterswijk, C.; Giselsbrecht, S.; Habibović, P.; Truckenmüller, R. K., In vitro vascularization of 3D cell aggregates in microwells with integrated vascular beds. *Materials Today Bio* **2024**, 29, 101260.
- Carvalho, D. T. O.; Feijão, T.; Neves, M. I.; da Silva, R. M. P.; Barrias, C. C., Directed self-assembly of spheroids into modular vascular beds for engineering large tissue constructs. *Biofabrication* **2021**, 13 (3).
- Xiaolei, N.; Hashimoto, M., Fabrication and characterization of photopatterned 3D cell-laden grafts. *Materials Today: Proceedings* **2022**, 70, 143-147.
- Wu, K.-W.; Kuo, C.-T.; Tu, T.-Y., A Highly Reproducible Micro U-Well Array Plate Facilitating High-Throughput Tumor Spheroid Culture and Drug Assessment. *Global Challenges* **2021**, 5 (2), 2000056.
- Feijao, T.; Neves, M. I.; Sousa, A.; Torres, A. L.; Bidarra, S. J.; Orge, I. D.; Carvalho, D. T. O.; Barrias, C. C., Engineering injectable vascularized tissues from the bottom-up: Dynamics of in-gel extra-spheroid dermal tissue assembly. *Biomaterials* **2021**, 279, 121222.

21. Orge, I. D.; Nogueira Pinto, H.; Silva, M. A.; Bidarra, S. J.; Ferreira, S. A.; Calejo, I.; Masereeuw, R.; Mihăilă, S. M.; Barrias, C. C., Vascular units as advanced living materials for bottom-up engineering of perfusable 3D microvascular networks. *Bioactive Materials* **2024**, *38*, 499-511.
22. Minne, M.; Terrie, L.; Wüst, R.; Hasevoets, S.; Vanden Kerchove, K.; Nimako, K.; Lambrechts, I.; Thorrez, L.; Declercq, H., Generating human skeletal myoblast spheroids for vascular myogenic tissue engineering. *Biofabrication* **2024**, *16* (2), 025035.
23. Kim, E. M.; Lee, Y. B.; Kim, S.-j.; Park, J.; Lee, J.; Kim, S. W.; Park, H.; Shin, H., Fabrication of core-shell spheroids as building blocks for engineering 3D complex vascularized tissue. *Acta Biomaterialia* **2019**, *100*, 158-172.
24. Wei, Z.; Lei, M.; Wang, Y.; Xie, Y.; Xie, X.; Lan, D.; Jia, Y.; Liu, J.; Ma, Y.; Cheng, B.; Gerecht, S.; Xu, F., Hydrogels with tunable mechanical plasticity regulate endothelial cell outgrowth in vasculogenesis and angiogenesis. *Nature Communications* **2023**, *14* (1), 8307.
25. Liu, Y.; Zhang, Y.; Mei, T.; Cao, H.; Hu, Y.; Jia, W.; Wang, J.; Zhang, Z.; Wang, Z.; Le, W.; Liu, Z., hESCs-Derived Early Vascular Cell Spheroids for Cardiac Tissue Vascular Engineering and Myocardial Infarction Treatment. *Adv Sci (Weinh)* **2022**, *9* (9), e2104299.
26. Hong, G.; Kim, J.; Oh, H.; Yun, S.; Kim, C. M.; Jeong, Y.-M.; Yun, W.-S.; Shim, J.-H.; Jang, I.; Kim, C.-Y.; Jin, S., Production of Multiple Cell-Laden Microtissue Spheroids with a Biomimetic Hepatic-Lobule-Like Structure. *Advanced Materials* **2021**, *33* (36), 2102624.
27. Liu, C.; Chen, Y.; Chen, H.; Zhang, P., Droplet-based bioprinting for fabrication of tumor spheroids. *IJB* **2024**, *10* (1).
28. Son, K. H.; Kim, D.-H.; Park, S.; Kim, H. J.; Park, M.; Kim, S.-J.; Lee, S. J.; Ahn, K.; Lee, J. W., Spherical Shell Bioprinting to Produce Uniform Spheroids with Controlled Sizes. *Journal of Functional Biomaterials* **2024**, *15* (11), 350.
29. Lee, S. I.; Choi, Y. Y.; Kang, S. G.; Kim, T. H.; Choi, J. W.; Kim, Y. J.; Kim, T.-H.; Kang, T.; Chung, B. G., 3D Multicellular Tumor Spheroids in a Microfluidic Droplet System for Investigation of Drug Resistance. *Polymers* **2022**, *14* (18), 3752.
30. Chen, P.; Miao, Y.; Zhang, F.; Fan, Z.; Huang, J.; Mao, X.; Chen, J.; Hu, Z.; Wang, J., Tissue engineering ECM-enriched controllable vascularized human microtissue for hair regenerative medicine using a biomimetic developmental approach. *Journal of Advanced Research* **2022**, *38*, 77-89.
31. Wei, J.; Sun, Y.; Wang, H.; Zhu, T.; Li, L.; Zhou, Y.; Liu, Q.; Dai, Z.; Li, W.; Yang, T.; Wang, B.; Zhu, C.; Shen, X.; Yao, Q.; Song, G.; Zhao, Y.; Pei, H., Designer cellular spheroids with DNA origami for drug screening. *Science Advances* **2024**, *10* (29), eado9880.
32. Dejana, E.; Hirschi, K. K.; Simons, M., The molecular basis of endothelial cell plasticity. *Nature Communications* **2017**, *8* (1), 14361.
33. Chi, J.-T.; Chang, H. Y.; Haraldsen, G.; Jahnsen, F. L.; Troyanskaya, O. G.; Chang, D. S.; Wang, Z.; Rockson, S. G.; van de Rijn, M.; Botstein, D.; Brown, P. O., Endothelial cell diversity revealed by global expression profiling. *Proceedings of the National Academy of Sciences* **2003**, *100* (19), 10623-10628.
34. Kalucka, J.; de Rooij, L. P. M. H.; Goveia, J.; Rohlenova, K.; Dumas, S. J.; Meta, E.; Conchinha, N. V.; Taverna, F.; Teuwen, L.-A.; Veys, K.; García-Caballero, M.; Khan, S.; Geldhof, V.; Sokol, L.; Chen, R.; Treps, L.; Borri, M.; de Zeeuw, P.; Dubois, C.; Karakach, T. K.; Falkenberg, K. D.; Parys, M.; Yin, X.; Vinckier, S.; Du, Y.; Fenton, R. A.; Schoonjans, L.; Dewerchin, M.; Eelen, G.; Thienpont, B.; Lin, L.; Bolund, L.; Li, X.; Luo, Y.; Carmeliet, P., Single-Cell Transcriptome Atlas of Murine Endothelial Cells. *Cell* **2020**, *180* (4), 764-779.e20.
35. Urbanczyk, M.; Zbinden, A.; Schenke-Layland, K., Organ-specific endothelial cell heterogeneity and its impact on regenerative medicine and biomedical engineering applications. *Advanced Drug Delivery Reviews* **2022**, *186*, 114323.
36. Augustin, H. G.; Koh, G. Y., A systems view of the vascular endothelium in health and disease. *Cell* **2024**, *187* (18), 4833-4858.
37. Aghazadeh, Y.; Khan, S. T.; Nkenkor, B.; Nunes, S. S., Cell-based therapies for vascular regeneration: Past, present and future. *Pharmacology & Therapeutics* **2022**, *231*, 107976.
38. Dienemann, S.; Schmidt, V.; Fleischhammer, T.; Mueller, J. H.; Lavrentieva, A., Comparative analysis of hypoxic response of human microvascular and umbilical vein endothelial cells in 2D and 3D cell culture systems. *Journal of Cellular Physiology* **2023**, *238* (5), 1111-1120.
39. March, S.; Hui, E. E.; Underhill, G. H.; Khetani, S.; Bhatia, S. N., Microenvironmental regulation of the sinusoidal endothelial cell phenotype in vitro. *Hepatology* **2009**, *50* (3), 920-8.
40. Sundaram, S.; Chen, C. S., Next-generation engineered microsystems for cell biology: a systems-level roadmap. *Trends in Cell Biology* **2022**, *32* (6), 490-500.

41. Ingram, D. A.; Mead, L. E.; Tanaka, H.; Meade, V.; Fenoglio, A.; Mortell, K.; Pollok, K.; Ferkowicz, M. J.; Gilley, D.; Yoder, M. C., Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. *Blood* **2004**, *104* (9), 2752-60.
42. Curtis, M. B.; Kelly, N.; Hughes, C. C. W.; George, S. C., Organotypic stromal cells impact endothelial cell transcriptome in 3D microvessel networks. *Scientific Reports* **2022**, *12* (1), 20434.
43. Bauman, E.; Feijão, T.; Carvalho, D. T. O.; Granja, P. L.; Barrias, C. C., Xeno-free pre-vascularized spheroids for therapeutic applications. *Scientific Reports* **2018**, *8* (1), 230.
44. Orge, I. D.; Nogueira Pinto, H.; Silva, M. A.; Bidarra, S. J.; Ferreira, S. A.; Calejo, I.; Masereeuw, R.; Mihăilă, S. M.; Barrias, C. C., Vascular units as advanced living materials for bottom-up engineering of perfusable 3D microvascular networks. *Bioact Mater* **2024**, *38*, 499-511.
45. Blatchley, M. R.; Gerecht, S., Reconstructing the Vascular Developmental Milieu In Vitro. *Trends in Cell Biology* **2020**, *30* (1), 15-31.
46. Low, L. A.; Mummery, C.; Berridge, B. R.; Austin, C. P.; Tagle, D. A., Organs-on-chips: into the next decade. *Nature Reviews Drug Discovery* **2021**, *20* (5), 345-361.
47. Nguyen, J.; Lin, Y.-Y.; Gerecht, S., The next generation of endothelial differentiation: Tissue-specific ECs. *Cell Stem Cell* **2021**, *28* (7), 1188-1204.
48. Barrs, R. W.; Jia, J.; Silver, S. E.; Yost, M.; Mei, Y., Biomaterials for Bioprinting Microvasculature. *Chemical Reviews* **2020**, *120* (19), 10887-10949.
49. Bezenah, J. R.; Kong, Y. P.; Putnam, A. J., Evaluating the potential of endothelial cells derived from human induced pluripotent stem cells to form microvascular networks in 3D cultures. *Scientific Reports* **2018**, *8* (1), 2671.
50. Kusuma, S.; Shen, Y.-I.; Hanjaya-Putra, D.; Mali, P.; Cheng, L.; Gerecht, S., Self-organized vascular networks from human pluripotent stem cells in a synthetic matrix. *Proceedings of the National Academy of Sciences* **2013**, *110* (31), 12601-12606.
51. Margariti, A.; Winkler, B.; Karamariti, E.; Zampetaki, A.; Tsai, T. N.; Baban, D.; Ragoussis, J.; Huang, Y.; Han, J. D.; Zeng, L.; Hu, Y.; Xu, Q., Direct reprogramming of fibroblasts into endothelial cells capable of angiogenesis and reendothelialization in tissue-engineered vessels. *Proc Natl Acad Sci U S A* **2012**, *109* (34), 13793-8.
52. Jiang, Z.; Xu, Y.; Fu, M.; Zhu, D.; Li, N.; Yang, G., Genetically modified cell spheroids for tissue engineering and regenerative medicine. *Journal of Controlled Release* **2023**, *354*, 588-605.
53. Orlova, V. V.; van den Hil, F. E.; Petrus-Reurer, S.; Drabsch, Y.; ten Dijke, P.; Mummery, C. L., Generation, expansion and functional analysis of endothelial cells and pericytes derived from human pluripotent stem cells. *Nature Protocols* **2014**, *9* (6), 1514-1531.
54. Au, P.; Tam, J.; Fukumura, D.; Jain, R. K., Bone marrow-derived mesenchymal stem cells facilitate engineering of long-lasting functional vasculature. *Blood* **2008**, *111* (9), 4551-4558.
55. Wang, Y.; Fang, J.; Liu, B.; Shao, C.; Shi, Y., Reciprocal regulation of mesenchymal stem cells and immune responses. *Cell Stem Cell* **2022**, *29* (11), 1515-1530.
56. LeBleu, V. S.; Neilson, E. G., Origin and functional heterogeneity of fibroblasts. *Faseb j* **2020**, *34* (3), 3519-3536.
57. Lee, U.; Zhang, Y.; Zhu, Y.; Luo, A. C.; Gong, L.; Tremmel, D. M.; Kim, Y.; Villarreal, V. S.; Wang, X.; Lin, R.-Z.; Cui, M.; Ma, M.; Yuan, K.; Wang, K.; Chen, K.; Melero-Martin, J. M., Robust differentiation of human pluripotent stem cells into mural progenitor cells via transient activation of NKX3.1. *Nature Communications* **2024**, *15* (1), 8392.
58. Whelan, I. T.; Moeendarbary, E.; Hoey, D. A.; Kelly, D. J., Biofabrication of vasculature in microphysiological models of bone. *Biofabrication* **2021**, *13* (3), 032004.
59. Burdis, R.; Kelly, D. J., Biofabrication and bioprinting using cellular aggregates, microtissues and organoids for the engineering of musculoskeletal tissues. *Acta Biomaterialia* **2021**, *126*, 1-14.
60. Zhuang, R. Z.; Lock, R.; Liu, B.; Vunjak-Novakovic, G., Opportunities and challenges in cardiac tissue engineering from an analysis of two decades of advances. *Nature Biomedical Engineering* **2022**, *6* (4), 327-338.
61. Gough, A.; Soto-Gutierrez, A.; Verneti, L.; Ebrahimkhani, M. R.; Stern, A. M.; Taylor, D. L., Human biomimetic liver microphysiology systems in drug development and precision medicine. *Nat Rev Gastroenterol Hepatol* **2021**, *18* (4), 252-268.
62. Rouleau, N.; Murugan, N. J.; Kaplan, D. L., Functional bioengineered models of the central nervous system. *Nature Reviews Bioengineering* **2023**.
63. DePalma, T. J.; Sivakumar, H.; Skardal, A., Strategies for developing complex multi-component in vitro tumor models: Highlights in glioblastoma. *Advanced Drug Delivery Reviews* **2022**, *180*, 114067.

64. Curvello, R.; Kast, V.; Ordóñez-Morán, P.; Mata, A.; Loessner, D., Biomaterial-based platforms for tumour tissue engineering. *Nature Reviews Materials* **2023**.
65. Monteiro, M. V.; Zhang, Y. S.; Gaspar, V. M.; Mano, J. F., 3D-bioprinted cancer-on-a-chip: level-up organotypic in vitro models. *Trends in Biotechnology* **2022**, *40* (4), 432-447.
66. Kim, H.; Kim, B.; Kim, S. J.; Choi, Y.; Kim, I. H.-R.; Han, J.; Park, Y.-G.; Han, Y.-M.; Park, J.-K., Reconfigurable Hanging Drop Microarray Platform for On-Demand Preparation and Analysis of Spheroid Array. *Advanced Healthcare Materials* **2024**, *n/a* (n/a), 2400501.
67. Kwon, H.; Lee, S.; Byun, H.; Huh, S. J.; Lee, E.; Kim, E.; Lee, J.; Shin, H., Engineering pre-vascularized 3D tissue and rapid vascular integration with host blood vessels via co-cultured spheroids-laden hydrogel. *Biofabrication* **2024**, *16* (2), 025029.
68. Vorwald, C. E.; Joshee, S.; Leach, J. K., Spatial localization of endothelial cells in heterotypic spheroids influences Notch signaling. *Journal of Molecular Medicine* **2020**, *98* (3), 425-435.
69. Ascheid, D.; Baumann, M.; Pinnecker, J.; Friedrich, M.; Szi-Marton, D.; Medved, C.; Bundalo, M.; Ortmann, V.; Öztürk, A.; Nandigama, R.; Hemmen, K.; Ergün, S.; Zernecke, A.; Hirth, M.; Heinze, K. G.; Henke, E., A vascularized breast cancer spheroid platform for the ranked evaluation of tumor microenvironment-targeted drugs by light sheet fluorescence microscopy. *Nature Communications* **2024**, *15* (1), 3599.
70. Bauman, E.; Feijao, T.; Carvalho, D. T. O.; Granja, P. L.; Barrias, C. C., Xeno-free pre-vascularized spheroids for therapeutic applications. *Sci Rep* **2018**, *8* (1), 230.
71. Lu, C.; Jin, A.; Liu, H.; Gao, C.; Sun, W.; Zhang, Y.; Dai, Q.; Liu, Y., Advancing tissue engineering through vascularized cell spheroids: building blocks of the future. *Biomaterials Science* **2025**, *13* (8), 1901-1922.
72. Caprio, N. D.; Burdick, J. A., Engineered biomaterials to guide spheroid formation, function, and fabrication into 3D tissue constructs. *Acta Biomater* **2023**, *165*, 4-18.
73. Bonanini, F.; Dinkelberg, R.; Torregrosa, M. C.; Kortekaas, N.; Hagens, T. M. S.; Treillard, S.; Kurek, D.; van Duinen, V.; Vulto, P.; Bircsak, K., A microvascularized in vitro liver model for disease modeling and drug discovery. *Biofabrication* **2024**, *17* (1).
74. Bonanini, F.; Kurek, D.; Previdi, S.; Nicolas, A.; Hendriks, D.; de Ruyter, S.; Meyer, M.; Clapés Cabrer, M.; Dinkelberg, R.; García, S. B.; Kramer, B.; Olivier, T.; Hu, H.; López-Iglesias, C.; Schavemaker, F.; Walinga, E.; Dutta, D.; Queiroz, K.; Domansky, K.; Ronden, B.; Joore, J.; Lanz, H. L.; Peters, P. J.; Trietsch, S. J.; Clevers, H.; Vulto, P., In vitro grafting of hepatic spheroids and organoids on a microfluidic vascular bed. *Angiogenesis* **2022**, *25* (4), 455-470.
75. Golebiowska, A. A.; Intravaia, J. T.; Sathe, V. M.; Kumbar, S. G.; Nukavarapu, S. P., Decellularized extracellular matrix biomaterials for regenerative therapies: Advances, challenges and clinical prospects. *Bioactive Materials* **2024**, *32*, 98-123.
76. Garreta, E.; Moya-Rull, D.; Marco, A.; Amato, G.; Ullate-Agote, A.; Tarantino, C.; Gallo, M.; Esporrín-Ubieto, D.; Centeno, A.; Vilas-Zornoza, A.; Mestre, R.; Kalil, M.; Gorroñogoitia, I.; Zaldua, A. M.; Sanchez, S.; Izquierdo Reyes, L.; Fernández-Santos, M. E.; Prosper, F.; Montserrat, N., Natural Hydrogels Support Kidney Organoid Generation and Promote In Vitro Angiogenesis. *Adv Mater* **2024**, e2400306.
77. Im, G. B.; Lin, R. Z., Bioengineering for vascularization: Trends and directions of photocrosslinkable gelatin methacrylate hydrogels. *Front Bioeng Biotechnol* **2022**, *10*, 1053491.
78. Escudero, M.; Vaysse, L.; Eke, G.; Peyrou, M.; Villarroya, F.; Bonnel, S.; Jeanson, Y.; Boyer, L.; Vieu, C.; Chaput, B.; Yao, X.; Deschaseaux, F.; Parny, M.; Raymond-Letron, I.; Dani, C.; Carrière, A.; Malaquin, L.; Casteilla, L., Scalable Generation of Pre-Vascularized and Functional Human Beige Adipose Organoids. *Adv Sci (Weinh)* **2023**, *10* (31), e2301499.
79. Friend, N. E.; Zhang, I. W.; Hu, M. M.; McCoy, A. J.; Kent III, R. N.; DePalma, S. J.; Baker, B. M.; Leshner-Pérez, S. C.; Stegemann, J. P.; Putnam, A. J., Biofabrication and Characterization of Vascularizing PEG-Norbornene Microgels. *Journal of Biomedical Materials Research Part A* **2025**, *113* (4), e37900.
80. Brown, A.; He, H.; Trumper, E.; Valdez, J.; Hammond, P.; Griffith, L. G., Engineering PEG-based hydrogels to foster efficient endothelial network formation in free-swelling and confined microenvironments. *Biomaterials* **2020**, *243*, 119921.
81. Chapla, R.; West, J. L., Hydrogel biomaterials to support and guide vascularization. *Progress in Biomedical Engineering* **2021**, *3* (1), 012002.
82. Thai, V. L.; Ramos-Rodriguez, D. H.; Mesfin, M.; Leach, J. K., Hydrogel degradation promotes angiogenic and regenerative potential of cell spheroids for wound healing. *Materials Today Bio* **2023**, *22*, 100769.

83. He, Y. J.; Santana, M. F.; Moucka, M.; Quirk, J.; Shuaibi, A.; Pimentel, M. B.; Grossman, S.; Rashid, M. M.; Cinar, A.; Georgiadis, J. G.; Vaicik, M. K.; Kawaji, K.; Venerus, D. C.; Papavasiliou, G., Immobilized RGD concentration and proteolytic degradation synergistically enhance vascular sprouting within hydrogel scaffolds of varying modulus. *J Biomater Sci Polym Ed* **2020**, *31* (3), 324-349.
84. Ayan, B.; Heo, D. N.; Zhang, Z.; Dey, M.; Povilianskas, A.; Drapaca, C.; Ozbolat, I. T., Aspiration-assisted bioprinting for precise positioning of biologics. *Science Advances* **2020**, *6* (10), eaaw5111.
85. Kim, S.-j.; Byun, H.; Lee, S.; Kim, E.; Lee, G. M.; Huh, S. J.; Joo, J.; Shin, H., Spatially arranged encapsulation of stem cell spheroids within hydrogels for the regulation of spheroid fusion and cell migration. *Acta Biomaterialia* **2022**, *142*, 60-72.
86. Kano, M. R.; Morishita, Y.; Iwata, C.; Iwasaka, S.; Watabe, T.; Ouchi, Y.; Miyazono, K.; Miyazawa, K., VEGF-A and FGF-2 synergistically promote neoangiogenesis through enhancement of endogenous PDGF-B-PDGFR β signaling. *Journal of Cell Science* **2005**, *118* (16), 3759-3768.
87. Pauty, J.; Usuba, R.; Cheng, I. G.; Hespel, L.; Takahashi, H.; Kato, K.; Kobayashi, M.; Nakajima, H.; Lee, E.; Yger, F.; Soncin, F.; Matsunaga, Y. T., A Vascular Endothelial Growth Factor-Dependent Sprouting Angiogenesis Assay Based on an In Vitro Human Blood Vessel Model for the Study of Anti-Angiogenic Drugs. *EBioMedicine* **2018**, *27*, 225-236.
88. Eelen, G.; de Zeeuw, P.; Simons, M.; Carmeliet, P., Endothelial cell metabolism in normal and diseased vasculature. *Circ Res* **2015**, *116* (7), 1231-44.
89. Rüttsche, D.; Nanni, M.; Rüdissler, S.; Biedermann, T.; Zenobi-Wong, M., Enzymatically Crosslinked Collagen as a Versatile Matrix for In Vitro and In Vivo Co-Engineering of Blood and Lymphatic Vasculature. *Advanced Materials* **2023**, *35* (16), 2209476.
90. Oliverio, R.; Liberelle, B.; Patenaude, V.; Moreau, V.; Thomas, E.; Virgilio, N.; Banquy, X.; De Crescenzo, G., Cofunctionalization of Macroporous Dextran Hydrogels with Adhesive Peptides and Growth Factors Enables Vascular Spheroid Sprouting. *ACS Biomaterials Science & Engineering* **2024**, *10* (8), 5080-5093.
91. Jo, Y.; Hwang, D. G.; Kim, M.; Yong, U.; Jang, J., Bioprinting-assisted tissue assembly to generate organ substitutes at scale. *Trends in Biotechnology* **2023**, *41* (1), 93-105.
92. Benmeridja, L.; De Moor, L.; De Maere, E.; Vanlauwe, F.; Ryx, M.; Tytgat, L.; Vercruysse, C.; Dubruel, P.; Van Vlierberghe, S.; Blondeel, P.; Declercq, H., High-throughput fabrication of vascularized adipose microtissues for 3D bioprinting. *Journal of Tissue Engineering and Regenerative Medicine* **2020**, *14* (6), 840-854.
93. De Moor, L.; Smet, J.; Plovyt, M.; Bekaert, B.; Vercruysse, C.; Asadian, M.; De Geyter, N.; Van Vlierberghe, S.; Dubruel, P.; Declercq, H., Engineering microvasculature by 3D bioprinting of prevascularized spheroids in photo-crosslinkable gelatin. *Biofabrication* **2021**, *13* (4).
94. Fang, Y.; Ji, M.; Wu, B.; Xu, X.; Wang, G.; Zhang, Y.; Xia, Y.; Li, Z.; Zhang, T.; Sun, W.; Xiong, Z., Engineering Highly Vascularized Bone Tissues by 3D Bioprinting of Granular Prevascularized Spheroids. *ACS Applied Materials & Interfaces* **2023**, *15* (37), 43492-43502.
95. Liu, X.; Wang, X.; Zhang, L.; Sun, L.; Wang, H.; Zhao, H.; Zhang, Z.; Liu, W.; Huang, Y.; Ji, S.; Zhang, J.; Li, K.; Song, B.; Li, C.; Zhang, H.; Li, S.; Wang, S.; Zheng, X.; Gu, Q., 3D Liver Tissue Model with Branched Vascular Networks by Multimaterial Bioprinting. *Advanced Healthcare Materials* **2021**, *10* (23), 2101405.
96. Park, Y.; Ji, S. T.; Yong, U.; Das, S.; Jang, W. B.; Ahn, G.; Kwon, S. M.; Jang, J., 3D bioprinted tissue-specific spheroidal multicellular microarchitectures for advanced cell therapy. *Biofabrication* **2021**, *13* (4).
97. Yu, T.; Yang, Q.; Peng, B.; Gu, Z.; Zhu, D., Vascularized organoid-on-a-chip: design, imaging, and analysis. *Angiogenesis* **2024**, *27* (2), 147-172.
98. Chrobak, K. M.; Potter, D. R.; Tien, J., Formation of perfused, functional microvascular tubes in vitro. *Microvasc Res* **2006**, *71* (3), 185-96.
99. Hospodiuk-Karwowski, M.; Chi, K.; Pritchard, J.; Catchmark, J. M., Vascularized pancreas-on-a-chip device produced using a printable simulated extracellular matrix. *Biomedical Materials* **2022**, *17* (6), 065006.
100. Quintard, C.; Tubbs, E.; Jonsson, G.; Jiao, J.; Wang, J.; Werschler, N.; Laporte, C.; Pitaval, A.; Bah, T.-S.; Pomeranz, G.; Bissardon, C.; Kaal, J.; Leopoldi, A.; Long, D. A.; Blandin, P.; Achard, J.-L.; Battail, C.; Hagelkruys, A.; Navarro, F.; Fouillet, Y.; Penninger, J. M.; Gidrol, X., A microfluidic platform integrating functional vascularized organoids-on-chip. *Nature Communications* **2024**, *15* (1), 1452.

101. Arslan, U.; Brescia, M.; Meraviglia, V.; Nahon, D. M.; van Helden, R. W. J.; Stein, J. M.; van den Hil, F. E.; van Meer, B. J.; Vila Cuenca, M.; Mummery, C. L.; Orlova, V. V., Vascularized hiPSC-derived 3D cardiac microtissue on chip. *Stem Cell Reports* **2023**.
102. Cio, S. D.; Haddrick, M.; Gautrot, J. E., Vascularised Cardiac Spheroids-on-a-Chip for Testing the Toxicity of Therapeutics. *Scientific Reports* **2023**, *14*, 3370.
103. Ahn, J.; Kim, D. H.; Koo, D. J.; Lim, J.; Park, T. E.; Lee, J.; Ko, J.; Kim, S.; Kim, M.; Kang, K. S.; Min, D. H.; Kim, S. Y.; Kim, Y.; Jeon, N. L., 3D microengineered vascularized tumor spheroids for drug delivery and efficacy testing. *Acta Biomater* **2023**, *165*, 153-167.
104. Dobos, A.; Gantner, F.; Markovic, M.; Van Hoorick, J.; Tytgat, L.; Van Vlierberghe, S.; Ovsianikov, A., On-chip high-definition bioprinting of microvascular structures. *Biofabrication* **2020**, *13* (1), 015016.
105. Lin, D. S. Y.; Rajasekar, S.; Marway, M. K.; Zhang, B., From Model System to Therapy: Scalable Production of Perfusable Vascularized Liver Spheroids in "Open-Top" 384-Well Plate. *ACS Biomaterials Science & Engineering* **2021**, *7* (7), 2964-2972.
106. Yang, J.; Lei, W.; Xiao, Y.; Tan, S.; Yang, J.; Lin, Y.; Yang, Z.; Zhao, D.; Zhang, C.; Shen, Z.; Hu, S., Generation of human vascularized and chambered cardiac organoids for cardiac disease modelling and drug evaluation. *Cell Proliferation* *n/a* (n/a), e13631.
107. Nashimoto, Y.; Okada, R.; Hanada, S.; Arima, Y.; Nishiyama, K.; Miura, T.; Yokokawa, R., Vascularized cancer on a chip: The effect of perfusion on growth and drug delivery of tumor spheroid. *Biomaterials* **2020**, *229*, 119547.
108. Hsu, T.-W.; Lu, Y.-J.; Lin, Y.-J.; Huang, Y.-T.; Hsieh, L.-H.; Wu, B.-H.; Lin, Y.-C.; Chen, L.-C.; Wang, H.-W.; Chuang, J.-C.; Fang, Y.-Q.; Huang, C.-C., Transplantation of 3D MSC/HUVEC spheroids with neuroprotective and proangiogenic potentials ameliorates ischemic stroke brain injury. *Biomaterials* **2021**, *272*, 120765.
109. Huang, C.-C.; Wei, H.-J.; Lin, K.-J.; Lin, W.-W.; Wang, C.-W.; Pan, W.-Y.; Hwang, S.-M.; Chang, Y.; Sung, H.-W., Multimodality noninvasive imaging for assessing therapeutic effects of exogenously transplanted cell aggregates capable of angiogenesis on acute myocardial infarction. *Biomaterials* **2015**, *73*, 12-22.
110. Chen, D.-Y.; Wei, H.-J.; Lin, K.-J.; Huang, C.-C.; Wang, C.-C.; Wu, C.-T.; Chao, K.-T.; Chen, K.-J.; Chang, Y.; Sung, H.-W., Three-dimensional cell aggregates composed of HUVECs and cbMSCs for therapeutic neovascularization in a mouse model of hindlimb ischemia. *Biomaterials* **2013**, *34* (8), 1995-2004.
111. Huang, C.-C.; Pan, W.-Y.; Tseng, M. T.; Lin, K.-J.; Yang, Y.-P.; Tsai, H.-W.; Hwang, S.-M.; Chang, Y.; Wei, H.-J.; Sung, H.-W., Enhancement of cell adhesion, retention, and survival of HUVEC/cbMSC aggregates that are transplanted in ischemic tissues by concurrent delivery of an antioxidant for therapeutic angiogenesis. *Biomaterials* **2016**, *74*, 53-63.
112. Bayarara, O.; Dashnyam, K.; Singh, R. K.; Mandakhbayar, N.; Lee, J. H.; Park, J.-T.; Lee, J.-H.; Kim, H.-W., Nanoceria-GO-intercalated multicellular spheroids revascularize and salvage critical ischemic limbs through anti-apoptotic and pro-angiogenic functions. *Biomaterials* **2023**, *292*, 121914.
113. Gonçalves, R. C.; Banfi, A.; Oliveira, M. B.; Mano, J. F., Strategies for re-vascularization and promotion of angiogenesis in trauma and disease. *Biomaterials* **2021**, *269*, 120628.
114. Heo, D. N.; Hospodiuk, M.; Ozbolat, I. T., Synergistic interplay between human MSCs and HUVECs in 3D spheroids laden in collagen/fibrin hydrogels for bone tissue engineering. *Acta Biomaterialia* **2019**, *95*, 348-356.
115. Bashor, C. J.; Hilton, I. B.; Bandukwala, H.; Smith, D. M.; Veisheh, O., Engineering the next generation of cell-based therapeutics. *Nature Reviews Drug Discovery* **2022**, *21* (9), 655-675.
116. García-Fernández, C.; López-Fernández, A.; Borrós, S.; Lecina, M.; Vives, J., Strategies for large-scale expansion of clinical-grade human multipotent mesenchymal stromal cells. *Biochemical Engineering Journal* **2020**, *159*, 107601.
117. Rivera-Ordaz, A.; Peli, V.; Manzini, P.; Barilani, M.; Lazzari, L., Critical Analysis of cGMP Large-Scale Expansion Process in Bioreactors of Human Induced Pluripotent Stem Cells in the Framework of Quality by Design. *BioDrugs* **2021**, *35* (6), 693-714.
118. Kropp, C.; Massai, D.; Zweigerdt, R., Progress and challenges in large-scale expansion of human pluripotent stem cells. *Process Biochemistry* **2017**, *59*, 244-254.
119. Sacchi, M.; Bansal, R.; Rouwkema, J., Bioengineered 3D Models to Recapitulate Tissue Fibrosis. *Trends Biotechnol* **2020**, *38* (6), 623-636.
120. Seidi, K.; Ayoubi-Joshaghani, M. H.; Azizi, M.; Javaheri, T.; Jaymand, M.; Alizadeh, E.; Webster, T. J.; Yazdi, A. A.; Niazi, M.; Hamblin, M. R.; Amoozgar, Z.; Jahanban-Esfahlan, R., Bioinspired hydrogels build a bridge from bench to bedside. *Nano Today* **2021**, *39*, 101157.

121. Vunjak-Novakovic, G.; Ronaldson-Bouchard, K.; Radisic, M., Organs-on-a-chip models for biological research. *Cell* **2021**, *184* (18), 4597-4611.
122. Jalili-Firoozinezhad, S.; Miranda, C. C.; Cabral, J. M. S., Modeling the Human Body on Microfluidic Chips. *Trends in Biotechnology* **2021**, *39* (8), 838-852.
123. Fritschen, A.; Blaeser, A., Biosynthetic, biomimetic, and self-assembled vascularized Organ-on-a-Chip systems. *Biomaterials* **2021**, *268*, 120556.
124. Zhao, Y.; Wang, E. Y.; Lai, F. B. L.; Cheung, K.; Radisic, M., Organs-on-a-chip: a union of tissue engineering and microfabrication. *Trends in Biotechnology* **2023**, *41* (3), 410-424.
125. Picollet-D'hahan, N.; Zuchowska, A.; Lemeunier, I.; Le Gac, S., Multiorgan-on-a-Chip: A Systemic Approach To Model and Decipher Inter-Organ Communication. *Trends in Biotechnology* **2021**, *39* (8), 788-810.
126. Fisher, L. E.; Kämmerling, L.; Alexander, M. R.; Ghaemmaghami, A. M., Immune-instructive materials as new tools for immunotherapy. *Current Opinion in Biotechnology* **2022**, *74*, 194-203.
127. Graney, P. L.; Ben-Shaul, S.; Landau, S.; Bajpai, A.; Singh, B.; Eager, J.; Cohen, A.; Levenberg, S.; Spiller, K. L., Macrophages of diverse phenotypes drive vascularization of engineered tissues. *Science Advances* **2020**, *6* (18), eaay6391.
128. Giustarini, G.; Teng, G.; Pavesi, A.; Adriani, G., Characterization of 3D heterocellular spheroids of pancreatic ductal adenocarcinoma for the study of cell interactions in the tumor immune microenvironment. *Frontiers in Oncology* **2023**, *13*, 1156769.
129. Lê, H.; Deforges, J.; Hua, G.; Idoux-Gillet, Y.; Ponté, C.; Lindner, V.; Olland, A.; Falcoz, P. E.; Zupa, C.; Jain, S.; Quéméneur, E.; Benkirane-Jessel, N.; Balloul, J. M., In vitro vascularized immunocompetent patient-derived model to test cancer therapies. *iScience* **2023**, *26* (10), 108094.
130. Bogseth, A.; Ramirez, A.; Vaughan, E.; Maisel, K., In Vitro Models of Blood and Lymphatic Vessels—Connecting Tissues and Immunity. *Advanced Biology* **2023**, *0* (n/a), 2200041.
131. Das, S.; Gordián-Vélez, W. J.; Ledebur, H. C.; Mourkoti, F.; Rompolas, P.; Chen, H. I.; Serruya, M. D.; Cullen, D. K., Innervation: the missing link for biofabricated tissues and organs. *npj Regenerative Medicine* **2020**, *5* (1), 11.
132. Ledo, A. M.; Senra, A.; Rilo-Alvarez, H.; Borrajo, E.; Vidal, A.; Alonso, M. J.; Garcia-Fuentes, M., mRNA-activated matrices encoding transcription factors as primers of cell differentiation in tissue engineering. *Biomaterials* **2020**, *247*, 120016.
133. Callaghan, N. I.; Durland, L. J.; Ireland, R. G.; Santerre, J. P.; Simmons, C. A.; Davenport Huyer, L., Harnessing conserved signaling and metabolic pathways to enhance the maturation of functional engineered tissues. *npj Regenerative Medicine* **2022**, *7* (1), 44.
134. Shariati, K.; Ling, A. S.; Fuchs, S.; Dillenburg, B.; Liu, W.; Ma, M., Hylozoic by Design: Converging Material and Biological Complexities for Cell-Driven Living Materials with 4D Behaviors. *Advanced Functional Materials* **2022**, *32* (7), 2108057.
135. Velazquez, J. J.; LeGraw, R.; Moghadam, F.; Tan, Y.; Kilbourne, J.; Maggiore, J. C.; Hislop, J.; Liu, S.; Cats, D.; Chuva de Sousa Lopes, S. M.; Plaisier, C.; Cahan, P.; Kiani, S.; Ebrahimkhani, M. R., Gene Regulatory Network Analysis and Engineering Directs Development and Vascularization of Multilineage Human Liver Organoids. *Cell Systems* **2021**, *12* (1), 41-55.e11.
136. Arjoca, S.; Robu, A.; Neagu, M.; Neagu, A., Mathematical and computational models in spheroid-based biofabrication. *Acta Biomaterialia* **2023**, *165*, 125-139.

Chapter II

Vascularized models of cholangiocyte organoids: an overview

1. The need for vascularization in organoid cultures

By definition, organoids are 3D structures derived from (pluripotent) stem cells, progenitor, and/or differentiated cells that self-organize through cell-cell and cell-matrix interactions to recapitulate aspects of the native tissue architecture and function *in vitro* ^[1]. Organoids have emerged as powerful models for studying human organ development, disease modelling, and regenerative medicine. However, their reliance on passive diffusion for nutrient and oxygen transport becomes increasingly inefficient as they grow in size. Additionally, inadequate waste removal presents a significant challenge in long-term cultures. These limitations often result in necrotic cores, impaired cell viability, and suboptimal differentiation, restricting their potential for applications in fundamental and translational research. Overcoming these challenges through induced vascularization is a promising approach for enhancing organoid functionality and expanding their utility as 3D *in vitro* models and as tools for regenerative medicine. Several strategies have been explored to integrate vascular networks into organoids, which can be broadly classified into six main categories: 1) co-culture with endothelial cells, 2) co-culture with pre-formed vascular microtissues (often called vascular organoids), 3) simultaneous differentiation of pluripotent stem cells into parenchymal and vascular/stromal cells, 4) microfluidic-based systems, 5) bioprinting techniques, and 6) *in vivo* implantation ^[2, 3]. These strategies are illustrated in Figure 1 and the main advantages and disadvantages of each one are briefly described on Table 1.

In addition to vascularization, increasing the overall complexity of organoid systems by incorporating key elements of the native tissue microenvironment – such as stromal cells, immune components, and tissue-specific extracellular matrix, can further enhance their structural fidelity and functional maturation. These components contribute not only to cellular diversity but also provide essential biochemical and biomechanical cues that regulate tissue development, homeostasis, and disease progression^[4]. Although such advanced multicellular and matrix-enriched approaches are not discussed in detail in this chapter, they represent a critical frontier in organoid research. By capturing the intricate interactions between different cell types and microenvironmental factors, these models are better equipped to replicate pathological processes, enabling more accurate drug screening, biomarker discovery, and therapeutic development. On the other hand, this increased physiological relevance may also facilitate their translation into regenerative medicine applications, where tissue integration, vascular support, and long-term function are crucial^[3].

Given the scope of this thesis, this chapter provides an overview of vascularized strategies focused on intrahepatic cholangiocyte organoids. These organoids were chosen as a principal model system due to relevance to biliary diseases, and amenability to integration with

engineered vascularized stroma. Their use enabled a deeper investigation into how a complex microenvironment influences tissue-specific differentiation and function/activation, offering valuable insights into both physiological and disease-relevant processes, such as hepatic fibrosis.

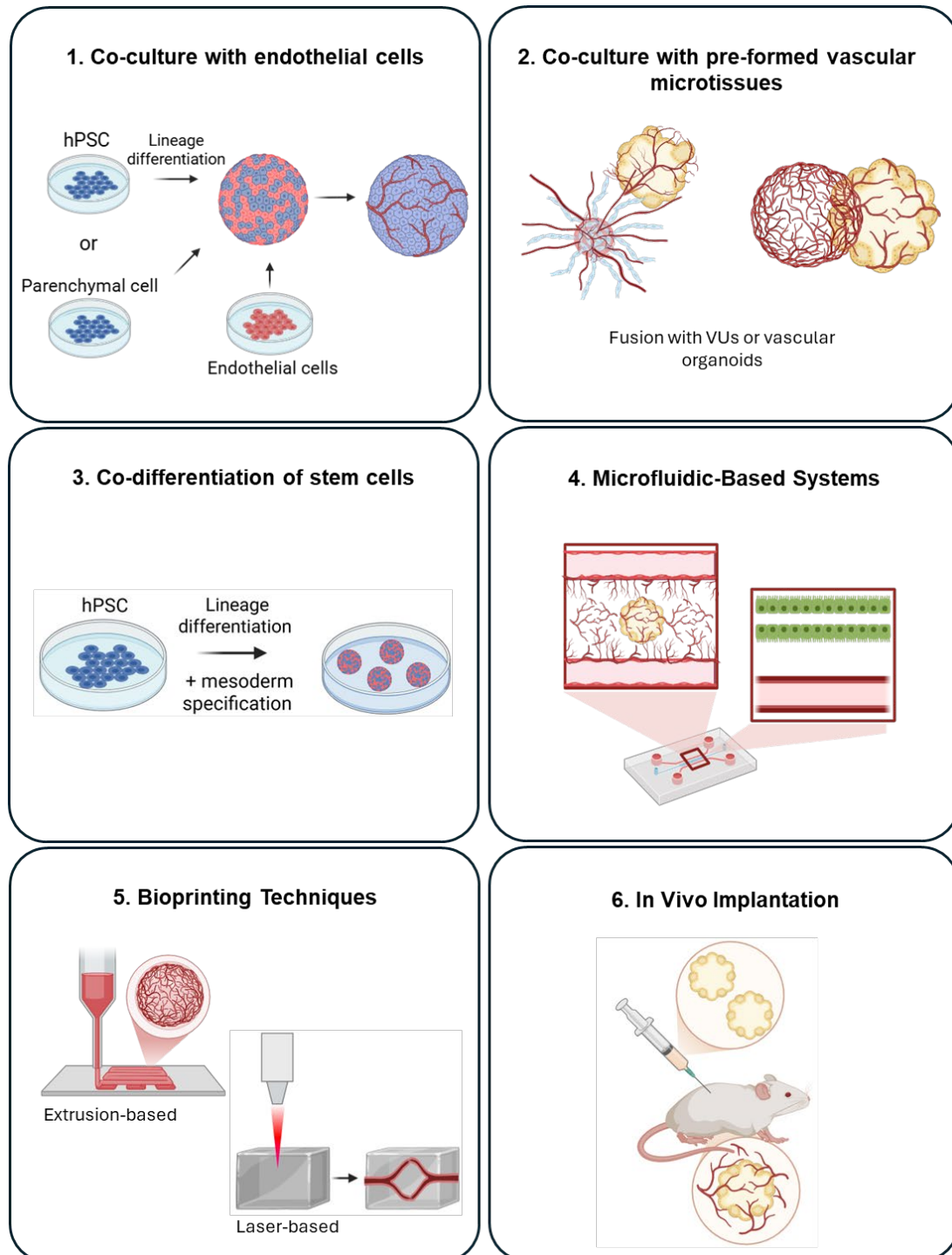


Figure 1. Schematic representation of different strategies for promoting organoid vascularization. Created with Biorender.com. Adapted from Nwokoye et al, 2024^[2].

Table 1. An overview of different strategies for promoting organoid vascularization.

Strategy	Advantages	Disadvantages	Applications	Ref.
Co-culture with endothelial cells	High scalability potential. Enhanced cellular crosstalk. Relatively simple to implement.	Limited nutrient diffusion and perfusability. Large number of cells needed to produce the structures. Difficulties in ensuring uniform vascularization in larger constructs.	Developmental studies. Disease modelling. Drug discovery. Personalized medicine.	[5-7]
Co-culture with pre-formed vascular microtissues	Physiological relevance. Faster vascularization. Enhanced tissue maturation. Better perfusion potential if integrated with microfluidic devices.	Standardization challenges as variability in the vascular network formation can affect culture compatibility with organoids. Limited perfusion without microfluidics. Challenging scale-up and high-throughput	Developmental studies. Disease modelling. Drug discovery. Personalized medicine.	[8-10]
Co-differentiation of stem cells	Intrinsic vascular integration: deeper tissue penetration and improved cellular crosstalk. All the cells could be derived of the same donor avoiding compatibility issues. Patient-derived iPSCs can recapitulate individual genetic backgrounds.	Lack of protocols standardization to generate mature cells. Complex and long differentiation protocols. Heterogeneity in vascularization. Lack of functional lumenization leading to limited perfusion.	Developmental studies. Disease modelling. Drug discovery. Personalized medicine.	[11, 12]
Microfluidic-based systems	Allows controlled perfusion and dynamic flow conditions. Enables endothelial cell patterning and vessel maturation. Promotes perfused vessels penetration within organoids. Integration of sensors for monitoring. Multiorgan systems	Requires specialized fabrication. Organoids integration can be technically demanding. Use of nonbiomimetic polymers (SL). Long-term culture maintenance can be challenging.	Developmental studies. Disease modelling. Drug discovery. Personalized medicine. Barrier function studies. Modelling angiogenesis and vasculogenesis.	[10, 13, 14]
Bioprinting techniques	High spatial control over vascular structure. Scalable for tissue engineering. Fast method. Multiple cell/materials	Low cell viability post-printing. Low resolution (extrusion). Cell sedimentation during printing can impair vessel formation. Limit mechanical properties	Developmental studies. Disease modelling. Drug discovery. Personalized medicine. Vascularized organoids for tissue grafting.	[15, 16]
In vivo implantation	Physiological vascularization via host integration. Functional blood perfusion possible.	Ethical and regulatory issues. Limited reproducibility. Not suitable for high-throughput studies.	Studying vascular integration in regenerative medicine. Disease modelling. Study of organoids engraftment and maturation in vivo.	[5, 17, 18]

2. Intrahepatic cholangiocyte organoids

Primary human cholangiocytes are ciliated epithelial cells that line the intrahepatic and extrahepatic bile ducts in the liver. These cells play a crucial role in bile production, modification, and transport, contributing to liver homeostasis and digestion. Moreover, they play a key role in liver repair, innate immunity, and progression of cholangiopathies, representing the first line of defence in the innate immunity of the liver ^[19]. When cultured in 2D set ups in vitro, these cells are known to be difficult to isolate and tend to de-differentiate after a few passages ^[20]. However, when embedded in matrix-rich 3D environment, such as Matrigel, primary cholangiocytes from intra or extra-hepatic bile ducts are capable to generate organoids ^[21].

Intrahepatic cholangiocytes organoids (ICOs) are epithelial organoids formed by EpCAM+ differentiated cells, named cholangiocytes, that undergo Tet1-mediated epigenetic reprogramming to assume a stem/progenitor cell state. Their typical morphology is characterized by cystic and polarized structures with long-term clonal expansion and high proliferative capacity, while remaining genetically stable. When cultured under the described conditions, ICOs express multiple progenitor, hepatocyte, and cholangiocyte markers, including LGR5, HNF4 α , KRT19 and KRT7, suggestive of a bipotential nature^[22]. When further differentiated towards matured cholangiocytes organoids, ICOs exhibit specific markers and functional characteristics indicative of their maturation as Multidrug Resistance Protein 1 (MDR1) high activity and increased expression of functional markers such as Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), Aquaporin 1 (AQP1), Aquaporin 4 (AQP4) and Gamma-Glutamyltransferase 1 (GGT1)^[23-25].

Additionally, unlike extrahepatic cholangiocyte organoids (ECOs), ICOs have the capacity to partially differentiate towards hepatocyte-like cells when cultured in specific conditions^[21], acquiring mature hepatocyte functions, such as albumin and bile acid secretion, glycogen storage, phase I and II drug metabolism, and ammonia detoxification^[1, 26].

Cholangiocytes' remarkable plasticity enables ICOs to serve as a powerful tool for studying both ductal and hepatocyte physiology in vitro. Furthermore, this inherent adaptability allows cholangiocytes to respond to physiological changes and actively contribute to liver regeneration in the face of injury or disease. The regulation of cholangiocyte-to-hepatocyte transdifferentiation is an area of ongoing research. However, studies suggest that this process is driven by the suppression of Notch and Wnt signalling, which enables biliary epithelial cells to lose their ductal identity and acquire hepatocyte-like characteristics^[27, 28].

In conclusion, cholangiocyte plasticity can be seen as a double-edged sword – while it plays a crucial role in supporting liver regeneration, it can also contribute to fibrosis and disease under chronic conditions. Excessive activation of cholangiocytes in such scenarios leads to bile duct scarring and liver dysfunction. Understanding these dynamic properties opens new opportunities for therapeutic strategies, particularly in organoid-based regenerative medicine and liver disease modelling.

3. Vascularized models of intrahepatic cholangiocyte organoids

Despite growing interest in organoid vascularization, relatively few strategies have been specifically developed for intrahepatic cholangiocyte organoids. Some approaches focus on integrating vascular components by co-culturing liver cells with endothelial and stromal cells in 3D systems, often through aggregation-based methods called “liver buds”. In these models, endothelial cells self-organize within the construct, forming primitive capillary-like networks that mimic early vasculature^[5, 6, 29, 30] (Figure 2).

An emerging approach in biliary tissue engineering involves creating tubular biliary organoids integrated into microfluidic devices. These cholangiocyte-lined structures can be developed using microfluidic-based methods, such as casting hollow channels by using microneedles within collagen-based hydrogels^[31, 32], or employing precise hydrogel patterning techniques like laser etching to fabricate instructive scaffolds on chips^[15]. Alternatively, membrane scaffolds can be utilized to support cell adhesion and the formation of polarized, hollow architectures^[23] (Figure 2). Notably, these constructs have demonstrated long-term stability, maintaining biliary organoid identity and exhibiting key cholangiocyte characteristics, including transport activities and the formation of primary cilia. In some of these studies, vascular and stromal compartments were incorporated by embedding stromal cells within collagen-based hydrogels and positioning vascular channels parallel to the cholangiocyte-containing channels.^[15, 32] However, it remains unclear whether the vascular channels in these models establish direct contact with the organoid-lined epithelial channels. Although both the endothelial and epithelial components are supported within the same hydrogel matrix, there is no evidence of angiogenic sprouting toward the organoid structures. This limitation hinders the establishment of physiologically relevant vascular integration.

On the other hand, microfluidic systems designed to promote microvascular network formation between lateral perfusion sources have been shown to enhance vessel infiltration into hepatic microtissues, facilitating more physiologically relevant vascular integration while preserving the microtissues' phenotypic and functional characteristics. Additionally, these systems provide a

valuable platform for disease modelling, enabling the study of conditions affecting vascular, stromal, or parenchymal compartments in an integrative manner ^[14, 33].

Together, these advances represent important steps toward engineering more physiologically relevant biliary models. However, current systems still fall short in fully recapitulating the complex spatial and functional interactions between cholangiocyte organoids and a dynamic vascularized stroma. As such, there remains substantial room for improvement in advancing the fidelity of in vitro platforms for biliary disease modelling.

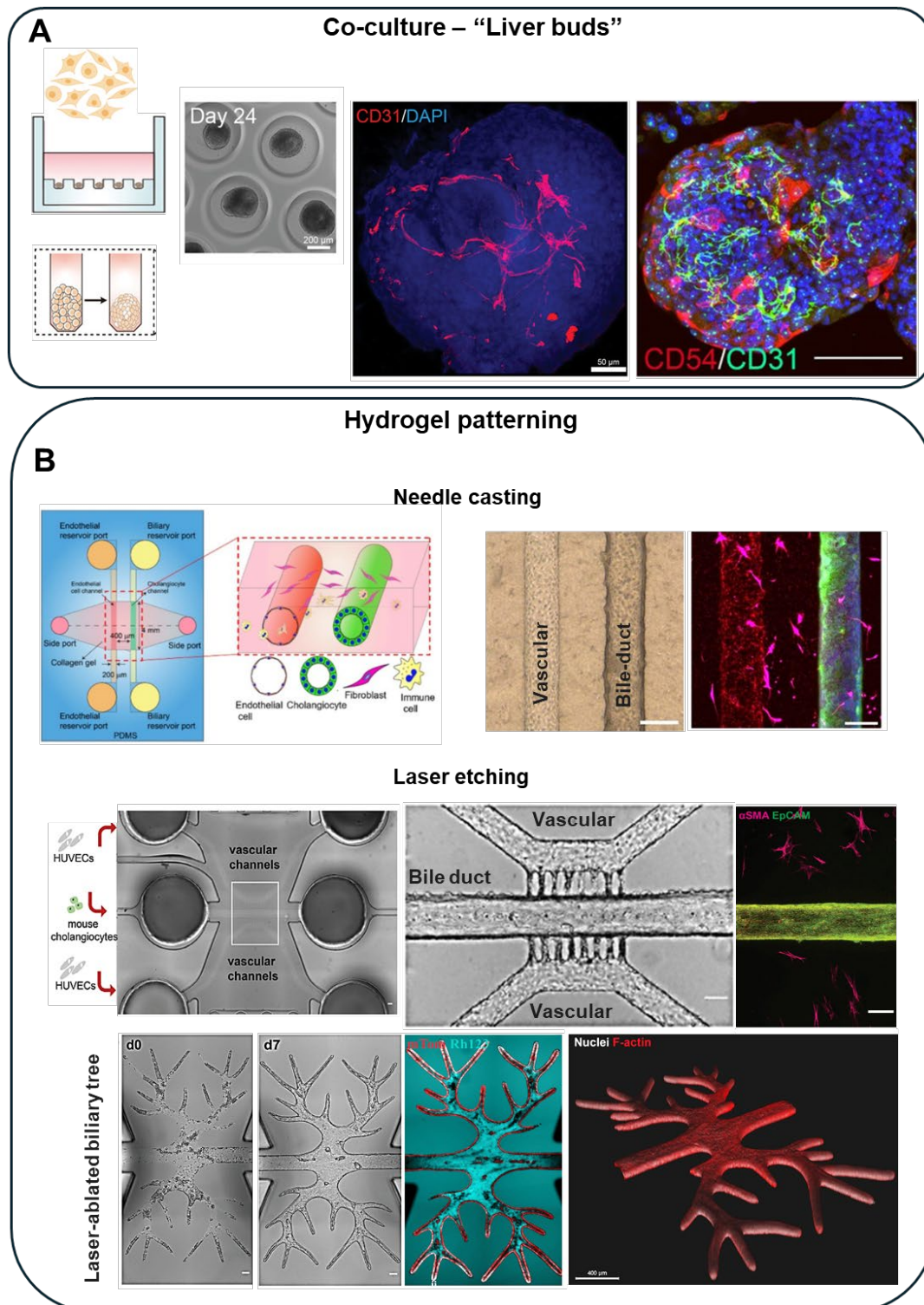


Figure 2. Strategies to incorporate vascular, stromal and/or immune compartments on cholangiocytes organoids. (A) Organoid development by aggregation of differentiated stem cells, endothelial cells and fibroblasts into spheroids called “liver buds”. Adapted from Fan *et al.* and Harrison *et al.* [34] [30] (Creative Commons CC BY licence). (B) Organoids engineered into bile duct channels using pre-patterning techniques, including needle molding and laser etching. In the first approach, the bile duct channel is positioned parallel to a vascular channel, with fibroblasts and immune cells embedded in the surrounding matrix. In the second approach, two vascular channels run parallel to the bile duct channel, allowing closer interaction through patterned protrusions toward the central duct. Laser-ablation technology also allowed the design of a biliary tree inside the chip. Adapted from Du *et al.* and Elci *et al.* [15, 32] (Creative Commons CC BY licence).

References

1. Marsee, A.; Roos, F. J. M.; Verstegen, M. M. A.; Gehart, H.; de Koning, E.; Lemaigre, F.; Forbes, S. J.; Peng, W. C.; Huch, M.; Takebe, T.; Vallier, L.; Clevers, H.; van der Laan, L. J. W.; Spee, B., Building consensus on definition and nomenclature of hepatic, pancreatic, and biliary organoids. *Cell Stem Cell* **2021**, *28* (5), 816-832.
2. Nwokoye, P. N.; Abilez, O. J., Bioengineering methods for vascularizing organoids. *Cell Reports Methods* **2024**, *4* (6), 100779.
3. Dellaquila, A.; Le Bao, C.; Letourneur, D.; Simon-Yarza, T., In Vitro Strategies to Vascularize 3D Physiologically Relevant Models. *Adv Sci (Weinh)* **2021**, *8* (19), e2100798.
4. Zeng, G.; Yu, Y.; Wang, M.; Liu, J.; He, G.; Yu, S.; Yan, H.; Yang, L.; Li, H.; Peng, X., Advancing cancer research through organoid technology. *J Transl Med* **2024**, *22* (1), 1007.
5. Takebe, T.; Sekine, K.; Enomura, M.; Koike, H.; Kimura, M.; Ogaeri, T.; Zhang, R. R.; Ueno, Y.; Zheng, Y. W.; Koike, N.; Aoyama, S.; Adachi, Y.; Taniguchi, H., Vascularized and functional human liver from an iPSC-derived organ bud transplant. *Nature* **2013**, *499* (7459), 481-4.
6. Camp, J. G.; Sekine, K.; Gerber, T.; Loeffler-Wirth, H.; Binder, H.; Gac, M.; Kanton, S.; Kageyama, J.; Damm, G.; Seehofer, D.; Belicova, L.; Bickle, M.; Barsacchi, R.; Okuda, R.; Yoshizawa, E.; Kimura, M.; Ayabe, H.; Taniguchi, H.; Takebe, T.; Treutlein, B., Multilineage communication regulates human liver bud development from pluripotency. *Nature* **2017**, *546* (7659), 533-538.
7. Yap, K. K.; Gerrand, Y.-W.; Dingle, A. M.; Yeoh, G. C.; Morrison, W. A.; Mitchell, G. M., Liver sinusoidal endothelial cells promote the differentiation and survival of mouse vascularised hepatobiliary organoids. *Biomaterials* **2020**, *251*, 120091.
8. Ahn, Y.; An, J. H.; Yang, H. J.; Lee, D. G.; Kim, J.; Koh, H.; Park, Y. H.; Song, B. S.; Sim, B. W.; Lee, H. J.; Lee, J. H.; Kim, S. U., Human Blood Vessel Organoids Penetrate Human Cerebral Organoids and Form a Vessel-Like System. *Cells* **2021**, *10* (8).
9. Garreta, E.; Moya-Rull, D.; Marco, A.; Amato, G.; Ullate-Agote, A.; Tarantino, C.; Gallo, M.; Esporrín-Ubieto, D.; Centeno, A.; Vilas-Zornoza, A.; Mestre, R.; Kalil, M.; Gorroñogoitia, I.; Zaldua, A. M.; Sanchez, S.; Izquierdo Reyes, L.; Fernández-Santos, M. E.; Prosper, F.; Montserrat, N., Natural Hydrogels Support Kidney Organoid Generation and Promote In Vitro Angiogenesis. *Adv Mater* **2024**, *36* (34), e2400306.
10. Orge, I. D.; Nogueira Pinto, H.; Silva, M. A.; Bidarra, S. J.; Ferreira, S. A.; Calejo, I.; Masereeuw, R.; Mihăilă, S. M.; Barrias, C. C., Vascular units as advanced living materials for bottom-up engineering of perfusable 3D microvascular networks. *Bioact Mater* **2024**, *38*, 499-511.
11. Abilez, O. J.; Yang, H.; Tian, L.; Wilson, K. D.; Lyall, E. H.; Shen, M.; Bhoi, R.; Zhuge, Y.; Jia, F.; Wo, H. T.; Zhou, G.; Guan, Y.; Aldana, B.; Obal, D.; Peltz, G.; Zarins, C. K.; Wu, J. C., Micropatterned Organoids Enable Modeling of the Earliest Stages of Human Cardiac Vascularization. *bioRxiv* **2022**, 2022.07.08.499233.
12. Cakir, B.; Xiang, Y.; Tanaka, Y.; Kural, M. H.; Parent, M.; Kang, Y. J.; Chapeton, K.; Patterson, B.; Yuan, Y.; He, C. S.; Raredon, M. S. B.; Dengelegi, J.; Kim, K. Y.; Sun, P.; Zhong, M.; Lee, S.; Patra, P.; Hyder, F.; Niklason, L. E.; Lee, S. H.; Yoon, Y. S.; Park, I. H., Engineering of human brain organoids with a functional vascular-like system. *Nat Methods* **2019**, *16* (11), 1169-1175.
13. Arslan, U.; Brescia, M.; Meraviglia, V.; Nahon, D. M.; van Helden, R. W. J.; Stein, J. M.; van den Hil, F. E.; van Meer, B. J.; Vila Cuenca, M.; Mummery, C. L.; Orlova, V. V., Vascularized hiPSC-derived 3D cardiac microtissue on chip. *Stem Cell Reports* **2023**, *18* (7), 1394-1404.
14. Bonanini, F.; Kurek, D.; Previdi, S.; Nicolas, A.; Hendriks, D.; de Ruiter, S.; Meyer, M.; Clapés Cabrer, M.; Dinkelberg, R.; García, S. B.; Kramer, B.; Olivier, T.; Hu, H.; López-Iglesias, C.; Schavemaker, F.; Walinga, E.; Dutta, D.; Queiroz, K.; Domansky, K.; Ronden, B.; Joore, J.; Lanz, H. L.; Peters, P. J.; Trietsch, S. J.; Clevers, H.; Vulto, P., In vitro grafting of hepatic spheroids and organoids on a microfluidic vascular bed. *Angiogenesis* **2022**, *25* (4), 455-470.
15. Elci, B. S.; Nikolaev, M.; Rezakhani, S.; Lutolf, M. P., Bioengineered Tubular Biliary Organoids. *Adv Healthc Mater* **2024**, *13* (8), e2302912.
16. Grebenyuk, S.; Abdel Fattah, A. R.; Kumar, M.; Toprakhisar, B.; Rustandi, G.; Vananroye, A.; Salmon, I.; Verfaillie, C.; Grillo, M.; Ranga, A., Large-scale perfused tissues via synthetic 3D soft microfluidics. *Nature Communications* **2023**, *14* (1), 193.
17. Freedman, B. S.; Dekel, B., Engraftment of Kidney Organoids In Vivo. *Curr Transplant Rep* **2023**, *10* (2), 29-39.
18. Kowalski, W. J.; Vatti, S.; Sakamoto, T.; Li, W.; Odutola, S. R.; Liu, C.; Chen, G.; Boehm, M.; Mukoyama, Y.-s., In vivo transplantation of mammalian vascular organoids onto the chick

- chorioallantoic membrane reveals the formation of a hierarchical vascular network. *Scientific Reports* **2025**, *15* (1), 7150.
19. Babboni, S.; Vacca, P. G.; Simonini, L.; Pezzati, D.; Martinelli, C.; Frongillo, F.; Bianco, G.; Marciano, E.; Basta, G.; Ghinolfi, D.; Del Turco, S., Cholangiocyte Organoids: The New Frontier in Regenerative Medicine for the Study and Treatment of Cholangiopathies. *J Clin Med* **2024**, *13* (6).
 20. Lancaster, M. A.; Knoblich, J. A., Organogenesis in a dish: modeling development and disease using organoid technologies. *Science* **2014**, *345* (6194), 1247125.
 21. Verstegen, M. M. A.; Roos, F. J. M.; Burka, K.; Gehart, H.; Jager, M.; de Wolf, M.; Bijvelds, M. J. C.; de Jonge, H. R.; Ardisasmita, A. I.; van Huizen, N. A.; Roest, H. P.; de Jonge, J.; Koch, M.; Pampaloni, F.; Fuchs, S. A.; Schene, I. F.; Luider, T. M.; van der Doef, H. P. J.; Bodewes, F. A. J. A.; de Kleine, R. H. J.; Spee, B.; Kremers, G.-J.; Clevers, H.; Ijzermans, J. N. M.; Cuppen, E.; van der Laan, L. J. W., Human extrahepatic and intrahepatic cholangiocyte organoids show region-specific differentiation potential and model cystic fibrosis-related bile duct disease. *Scientific Reports* **2020**, *10* (1), 21900.
 22. Huch, M.; Gehart, H.; van Boxtel, R.; Hamer, K.; Blokzijl, F.; Verstegen, M. M.; Ellis, E.; van Wenum, M.; Fuchs, S. A.; de Ligt, J.; van de Wetering, M.; Sasaki, N.; Boers, S. J.; Kemperman, H.; de Jonge, J.; Ijzermans, J. N.; Nieuwenhuis, E. E.; Hoekstra, R.; Strom, S.; Vries, R. R.; van der Laan, L. J.; Cuppen, E.; Clevers, H., Long-term culture of genome-stable bipotent stem cells from adult human liver. *Cell* **2015**, *160* (1-2), 299-312.
 23. Wang, Z.; Faria, J.; van der Laan, L. J. W.; Penning, L. C.; Masereeuw, R.; Spee, B., Human Cholangiocytes Form a Polarized and Functional Bile Duct on Hollow Fiber Membranes. *Front Bioeng Biotechnol* **2022**, *10*, 868857.
 24. Roos, F. J. M.; van Tienderen, G. S.; Wu, H.; Bordeu, I.; Vinke, D.; Albarinos, L. M.; Monfils, K.; Niesten, S.; Smits, R.; Willemse, J.; Rosmark, O.; Westergren-Thorsson, G.; Kunz, D. J.; de Wit, M.; French, P. J.; Vallier, L.; JNM, I. J.; Bartfai, R.; Marks, H.; Simons, B. D.; van Royen, M. E.; Verstegen, M. M. A.; van der Laan, L. J. W., Human branching cholangiocyte organoids recapitulate functional bile duct formation. *Cell Stem Cell* **2022**, *29* (5), 776-794.e13.
 25. Wang, Z.; Ye, S.; van der Laan, L. J. W.; Schneeberger, K.; Masereeuw, R.; Spee, B., Chemically Defined Organoid Culture System for Cholangiocyte Differentiation. *Adv Healthc Mater* **2024**, *13* (30), e2401511.
 26. Aizarani, N.; Saviano, A.; Sagar, Mailly, L.; Durand, S.; Herman, J. S.; Pessaux, P.; Baumert, T. F.; Grün, D., A human liver cell atlas reveals heterogeneity and epithelial progenitors. *Nature* **2019**, *572* (7768), 199-204.
 27. Pu, W.; Zhu, H.; Zhang, M.; Pikiolek, M.; Ercan, C.; Li, J.; Huang, X.; Han, X.; Zhang, Z.; Lv, Z.; Li, Y.; Liu, K.; He, L.; Liu, X.; Heim, M. H.; Terracciano, L. M.; Tchorz, J. S.; Zhou, B., Bipotent transitional liver progenitor cells contribute to liver regeneration. *Nature Genetics* **2023**, *55* (4), 651-664.
 28. Minnis-Lyons, S. E.; Ferreira-González, S.; Aleksieva, N.; Man, T. Y.; Gadd, V. L.; Williams, M. J.; Guest, R. V.; Lu, W.-Y.; Dwyer, B. J.; Jamieson, T.; Nixon, C.; Van Hul, N.; Lemaigre, F. P.; McCafferty, J.; Leclercq, I. A.; Sansom, O. J.; Boulter, L.; Forbes, S. J., Notch-IGF1 signaling during liver regeneration drives biliary epithelial cell expansion and inhibits hepatocyte differentiation. *Science Signaling* **2021**, *14* (688), eaay9185.
 29. Carolina, E.; Kuse, Y.; Okumura, A.; Aoshima, K.; Tadokoro, T.; Matsumoto, S.; Kanai, E.; Okumura, T.; Kasai, T.; Yamabe, S.; Nishikawa, Y.; Yamaguchi, K.; Furukawa, Y.; Tanimizu, N.; Taniguchi, H., Generation of human iPSC-derived 3D bile duct within liver organoid by incorporating human iPSC-derived blood vessel. *Nature Communications* **2024**, *15* (1), 7424.
 30. Harrison, S. P.; Siller, R.; Tanaka, Y.; Chollet, M. E.; de la Morena-Barrio, M. E.; Xiang, Y.; Patterson, B.; Andersen, E.; Bravo-Pérez, C.; Kempf, H.; Åsruud, K. S.; Lunov, O.; Dejneka, A.; Mowinckel, M. C.; Stavik, B.; Sandset, P. M.; Melum, E.; Baumgarten, S.; Bonanini, F.; Kurek, D.; Mathapati, S.; Almaas, R.; Sharma, K.; Wilson, S. R.; Skottvoll, F. S.; Boger, I. C.; Bogen, I. L.; Nyman, T. A.; Wu, J. J.; Bezrouk, A.; Cizkova, D.; Corral, J.; Mokry, J.; Zweigerdt, R.; Park, I. H.; Sullivan, G. J., Scalable production of tissue-like vascularized liver organoids from human PSCs. *Exp Mol Med* **2023**, *55* (9), 2005-2024.
 31. Chen, C.; Jochems, P. G. M.; Salz, L.; Schneeberger, K.; Penning, L. C.; van de Graaf, S. F. J.; Beuers, U.; Clevers, H.; Geijsen, N.; Masereeuw, R.; Spee, B., Bioengineered bile ducts recapitulate key cholangiocyte functions. *Biofabrication* **2018**, *10* (3), 034103.
 32. Du, Y.; de Jong, I. E. M.; Gupta, K.; Waisbourd-Zinman, O.; Har-Zahav, A.; Soroka, C. J.; Boyer, J. L.; Llewellyn, J.; Liu, C.; Naji, A.; Polacheck, W. J.; Wells, R. G., Human vascularized

- bile duct-on-a chip: a multi-cellular micro-physiological system for studying cholestatic liver disease. *Biofabrication* **2023**, *16* (1).
33. Bonanini, F.; Dinkelberg, R.; Torregrosa, M. C.; Kortekaas, N.; Hagens, T. M. S.; Treillard, S.; Kurek, D.; van Duinen, V.; Vulto, P.; Bircsak, K., A microvascularized in vitro liver model for disease modeling and drug discovery. *Biofabrication* **2024**, *17* (1).
 34. Fan, H.; Shang, J.; Li, J.; Yang, B.; Zhou, D.; Jiang, S.; Fan, Y.; Zhou, Y.; Wang, Y.; Liu, P.; Li, C.; Chen, Z.; Chen, P., High-Throughput Formation of Pre-Vascularized hiPSC-Derived Hepatobiliary Organoids on a Chip via Nonparenchymal Cell Grafting. *Advanced Science* *n/a* (n/a), 2407945.

Motivation and Aims

The development and application of 3D models of human tissues and organs, such as organoids, have gained substantial momentum over the past decade, emerging as powerful platforms for tissue engineering, disease modelling, and drug screening. Despite this progress, faithfully replicating the structural and functional complexity of human organs in vitro remains a significant challenge. Achieving higher levels of maturation and physiological relevance in organoid cultures requires the integration of key components of the stromal microenvironment, including microvessels, fibroblasts, and the ECM. This thesis focuses on the development of advanced vascularized 3D in vitro models engineered to recapitulate the dynamic interactions between vascular, stromal, and epithelial parenchymal compartments. The overarching objective was to create systems with enhanced physiological fidelity to advance our understanding of organogenesis, disease mechanisms, and therapeutic responses.

The specific aims were to:

- i. Develop organ-specific Vascular Units (VUs), i.e., microtissues composed of endothelial progenitor cells and organ-specific fibroblasts, as building blocks for the bottom-up assembly of stroma-embedded microvascular networks within a hydrogel-based matrix.
- ii. Evaluate the efficacy of a laminin-enriched, fibrin-based hydrogel in supporting the expansion and functional differentiation of organoids, compared to conventional basement membrane extract (BME)-based matrices. This approach aimed to provide a more defined, reproducible, and pro-angiogenic extracellular matrix environment.
- iii. Design and implement biomimetic, fibrin-based Vessel-on-Chip (VoC) platforms, enabling the formation of a perfusable and hierarchical microvascular network, along with stromal tissue, wrapping hydrogel-embedded organoids.
- iv. Utilize custom-designed biomimetic VoC platform to investigate how the presence of a fibrotic vascular-stromal microenvironment mimics pathological conditions of organ fibrosis and influences organoid response.

These aims were explored through two complementary studies, each employing a distinct organoid system: intestinal organoids (IO) and intrahepatic cholangiocyte organoids (ICOs). The first study primarily focused on the establishment of VUs and provided a preliminary evaluation of early vascularization of rapidly proliferating IOs. In contrast, the ICOs served as a model to explore how a fibrotic vascularized stroma impacts function in a more physiologically specialized hepatic context, particularly with relevance to biliary diseases. This enabled the validation of the VoC platform across different organ systems, highlighting its adaptability for modelling both homeostatic and disease-associated microenvironments.

Section B - Experimental Results

Chapter III

Vascular Units as Advanced Living Materials for Bottom-up Engineering of Perfusable 3D Microvascular Networks

Iasmim Orge^{1,2}, Henrique Nogueira Pinto¹, Marta Silva¹, Silvia Bidarra^{1,2}, Silvia A. Ferreira¹, Isabel Calejo¹, Ross Masereeuw⁴, Silvia Mihăilă⁴ and Cristina C. Barrias^{1,2,3}

¹ i3S – Instituto de Inovação e Investigação em Saúde, Rua Alfredo Allen, 208, 4200-135 Porto, Portugal

² INEB - Instituto de Engenharia Biomédica, Universidade do Porto, Portugal

³ ICBAS-Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Portugal

⁴ Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands.

ABSTRACT

The timely establishment of functional neo-vasculature is pivotal for successful tissue development and regeneration, remaining a central challenge in tissue engineering. In this study, we present a novel (micro)vascularization strategy that explores the use of specialized "vascular units" (VUs) as building blocks to initiate blood vessel formation and create perfusable, stroma-embedded 3D microvascular networks from the bottom-up. We demonstrate that VUs composed of endothelial progenitor cells and organ-specific fibroblasts exhibit high angiogenic potential when embedded in fibrin hydrogels. This leads to the formation of VUs-derived capillaries, which fuse with adjacent capillaries to form stable microvascular beds within a supportive, extracellular matrix-rich fibroblastic microenvironment. Using a custom-designed biomimetic fibrin-based vessel-on-chip (VoC), we show that VUs-derived capillaries can inosculate with endothelialized microfluidic channels in the VoC and become perfused. Moreover, VUs can establish capillary bridges between channels, extending the microvascular network throughout the entire device. When VUs and intestinal organoids (IOs) are combined within the VoC, the VUs-derived capillaries and the intestinal fibroblasts progressively reach and envelop the IOs. This promotes the formation of a supportive vascularized stroma around multiple IOs in a single device. These findings underscore the remarkable potential of VUs as building blocks for engineering microvascular networks, with versatile applications spanning from regenerative medicine to advanced in vitro models.

Keywords: engineered tissue, organ-on-chip, microtissue, spheroid, endothelial colony-forming cells.

1. Introduction

The presence of a well-developed vascular network in bioengineered tissues and organs is crucial for their functionality and applicability in therapeutic regeneration and disease modelling. The vascular system is among the first systems to develop during embryogenesis, as it is key to supporting and nourishing other tissues and organs^[1, 2]. It presents an intricate branching pattern, with larger diameter vessels giving rise to intricate networks of progressively smaller ones, ensuring that nearly every cell in the human body is located within a distance of only a few hundreds of micrometers from a capillary^[3]. Capillaries, the most prevalent vessels, are lined by a single layer of endothelial cells (EC), supported by basement membrane (BM) and pericytes, and their diameter typically ranges from 5 to 10 μm ^[4]. Capillary networks play a central role in tissue homeostasis, enabling the exchange of oxygen, carbon dioxide, nutrients, and metabolites between the bloodstream and the adjacent tissues. Therefore, establishing a functional (micro)vasculature down to the capillary scale in engineered tissues and organs is key to preserving cell viability and function, remaining a central challenge in regenerative medicine strategies.

The field of 3D bioprinting has witnessed significant progress in the ability to generate tubular structures with higher resolution and increased complexity, opening up new possibilities for fabricating blood vessel-like networks with precise architectures^[5, 6]. However, printing hollow structures at the capillary scale still represents a major challenge. Furthermore, in native tissues, the development of capillary beds follows a stochastic process, devoid of precise geometrical patterns, where the local microenvironment dynamically shapes ECs assembly into capillary networks that meet the unique metabolic demands of each organ^[7]. This highlights the complexity of microvascularization processes, suggesting that engineered capillary networks should ideally develop in situ, tailored to the specific context.

Harnessing living (micro)materials as building blocks for generating microvascular networks from the bottom-up offers an exciting approach toward this goal. In our prior studies we reported the ability to create capillary-like structures from vascularized microtissues composed of EC and dermal fibroblasts or mesenchymal stem cells^[8-10]. Expanding upon our previous findings, here we explored the use of vascularized microtissues (hereafter designated as Vascular Units, VUs) as versatile tools for generating perfusable stroma-embedded microvascular networks. We selected the small intestine as the target organ, as there is a pressing need for tissue engineering solutions in intestinal wound healing and a well-vascularized stroma is essential to support epithelial regeneration^[11]. Notably, considering that minimally invasive laparoscopic procedures currently set the standard in clinical interventions

for intestinal wounds, the micro-scale size of VUs positions them as an ideal system for this type of procedure ^[12].

We started by optimizing the biofabrication of VUs composed of human endothelial progenitors (endothelial colony-forming cells, ECFC) and organ-specific intestinal fibroblasts (hIF). ECFC presents high expansion potential and recognized ability to form de novo blood vessels in vivo, which makes them an ideal source for microvascular engineering ^[13]. On the other hand, fibroblasts act as supportive stromal cells, namely by producing extracellular matrix (ECM), which plays a key role in stabilizing the nascent microvessels, and may also act as pericytes ^[14]. The VUs were integrated in fibrin hydrogels to promote angiogenic sprouting and formation of lumenized endothelial networks. To better characterize their microvascularization potential, VUs were incorporated in a customized fibrin-based biomimetic vessel-on-chip (VoC) featuring endothelialized microfluidic channels with angiogenic sprouting potential. This allowed us to demonstrate the ability of VUs to inosculate (i.e., connect and integrate) with larger-diameter vessel-like microchannels and become perfused; to establish capillary bridges between channels; and to extend a stromal-embedded microvascular network throughout the entire device.

Finally, as a preliminary proof-of-concept, we demonstrated the applicability of VUs to promote the formation of a vascularized stroma in organoid cultures within the VoC. While organoids mimic important characteristics of native epitheliums, they still present limitations in achieving truly in vivo-like functionality. In particular, the absence of an external vascular supply and adequate stromal support may hinder proper growth, shorten lifespan, and impair functionality by preventing adequate differentiation and maturation ^[15]. Therefore, the development of effective strategies for organoid vascularization remains a challenge in the field ^[7, 16]. Here we show that the combination of VUs with IOs within the VOC offers a promising approach to promote the formation of a vascularized stroma around multiple organoids in one single microfluidic device. The formation of an ECM-rich fibroblastic stroma is per se relevant, as highly regenerative organs, like the intestine, strongly rely on fibroblast activity to maintain the stem cell niche ^[17].

Collectively, by providing versatile building blocks for in situ generation of perfusable microvascular beds embedded in stromal tissue, VUs offer a valuable tool for tissue engineering, with far-reaching implications in the fields of regenerative medicine, 3D in vitro models, and organ-on-chip (OoC).

2. Materials and Methods

2.1. Vascular units (VUs)

Cells sources and maintenance

Human umbilical cord blood was obtained from healthy donors following a protocol approved by the Ethics Committee of CHUSJ (Centro Hospitalar Universitário de São João, Porto, Portugal). Endothelial colony forming cells (ECFC) were isolated from umbilical cord blood as previously described ^[18-20]. All blood donors were kept anonymous, so the need for written consent was waived. Cells were maintained in ECFC growth medium composed of MCDB-131 basal medium (BM) supplemented with 5 % v/v fetal bovine serum (FBS), 1 % v/v of penicillin-streptomycin (Pen/Strep), 2 mM L-glutamine, 1 µg/mL ascorbic acid, 0.2 µg/mL hydrocortisone, 2 ng/mL VEGF, 20 ng/mL IGF-1R, 10 ng/mL FGF, and 5 ng/mL EGF. Cells were used at passages 5 to 8. Primary human intestinal fibroblasts (hIF) were acquired from Innoprot®. Cells were maintained on DMEM/F12 medium supplemented with 10% v/v FBS and 1% v/v Pen/Strep. Cells were used at passages 5 to 10. Both cell types were routinely cultured at 37 °C under a 5% v/v CO₂ humidified atmosphere and passage upon reaching ~80% confluence. The culture medium was changed every 2 days for ECFC and every 3 days for hIF.

Generation of vascular units (VUs)

VUs were generated using MicroTissues® 3D Petri Dish® technology as previously described ^[9]. Briefly, agarose-based micro-molds (2 % w/v agarose in 0.9 % w/v NaCl) were prepared using commercial templates (9x9 or 16x16 microwell arrays), placed in 12-well tissue culture plates and equilibrated with basal MCDB-131 medium overnight. ECFC and hIF were trypsinized and resuspended in their respective media. For 9x9 arrays, cells were seeded at a total density of 12000 cells/spheroid in 200 µL of EC growth medium at hIF:ECFC ratios of 1:1 (6000 hIF + 6000 ECFC) and 5:1 (10000 hIF + 2000 ECFC). For 16x16 arrays, cells were seeded at a total density of 6000 cells/spheroid in 200 µL of EC growth medium at hIF:ECFC ratio of 1:1 (3000 hIF + 3000 ECFC). Cells were allowed to settle for 30 min at room temperature (RT), and then additional medium was added to the wells. The medium was changed every 2 days. VUs were collected for analysis after 1, 5, and 7 days of culture. The metabolic activity of VUs was assessed with CellTiter-Glo® 3D Cell Viability Assay (ProMega) according to the manufacturer's instructions. Briefly, VUs were transferred from the mold to a white opaque-walled 96-well plate in 100 µL of growth medium (4 VUs per well) and 100 µL of CellTiter-Glo® 3D Reagent was added to each well. The plate was shaken in the microplate reader (Synergy MX, BioTek) for 5 min and incubated at room temperature for 25 min. After

incubation, luminescence was measured using a microplate reader (Synergy Mx, BioTek, integration time of 1 s, sensitivity of 135, top probe vertical offset of 1 mm).

Hydrogel embedding

Fibrin hydrogels were optimized according to previously published protocols [9, 10]. A fibrinogen solution (Sigma) was prepared in NaCl 0.9 % w/w mixed with aprotinin (Sigma) and diluted in EC growth medium to final concentrations of 2 mg/mL and 25 µ/mL, respectively. For VUs embedding, these were added to the fibrinogen solution, mixed with thrombin (2 U/mL in PBS) at 4:5 v/v ratio and left for 30 min at 37 °C for crosslinking.

Gene expression analysis

RNA was extracted from VUs using the Quick-RNA MiniPrep (Zymo Research), following the manufacturer instructions. Subsequently, 500 ng of total RNA was reversed transcribed to single stranded cDNA using the Takara cDNA synthesis kit. Quantitative Real-Time PCR (qRT-PCR) was carried out using source RNA from 3 biological replicates (i.e., 3 individual experiments) for the target genes *Col1a1*, *PECAM1*, *CDH5*, *LMNA*, *KDR*, *FLT1* and for endogenous control *GAPDH* using as probe sets Hs.PT.58.1551779, Hs.PT.58.19487865, Hs.PT.58.4732035, Hs.PT.58.27794, Hs.PT.58.3285240, Hs.PT.58.40906831 and Hs.PT.51.1940505 (Applied Biosystems and Integrated DNA Technologies), respectively. Samples were run in triplicates in the ABI Prism 7000 Sequence Detection System under the following conditions: 95 °C for 20 s, followed by 40 cycles at 95 °C for 3 s and 60 °C for 30 s. The expression value for each target gene was normalized to GAPDH value for all the samples.

Immunohistochemistry analysis of VUs

VUs were fixed inside the agarose micromolds with 4 % w/v PFA (30 min, RT). The molds were then sealed with 200 µL of molten HistoGel™ (ThermoFisher) and processed in an automatic rotational tissue processor (STP-120-1, MICROTOM). Samples were paraffin-embedded in an EC-350 embedding center and sectioned into 6 µm sections. Afterwards, sections were deparaffinized in xylene and rehydrated in sequentially decreasing ethanol concentrations. Antigen retrieval was performed with TE buffer (10 mM Tris, 1 mM EDTA, pH 9) or sodium citrate (10 mM, pH 6), at 95 °C for 30 min. Sections were permeabilized in 0.25 % v/v Triton X-100 (Sigma) for 10 min, blocked with 5% v/v FBS 1.5% w/v BSA in PBS for 1 h at room temperature and incubated overnight with the following primary antibodies diluted in blocking solution: mouse anti-CD31 (Dako, 1:100); rabbit anti-fibronectin (FN, Sigma, 1:200); rabbit anti-laminin (LAM, Sigma, 1:100); mouse anti-collagen type IV (COL IV, Dako, 1:100);

rabbit anti-collagen type I (COL I, Rockland, 1:100). Sections were then washed with PBS and incubated with the corresponding fluorochrome-conjugated secondary antibodies (Alexa-Invitrogen, 1:500) and 4',6-diamidino-2-phenylindole (DAPI, 1:1000) for 1 h at RT. Samples were washed and mounted with VectaShield (Vector).

Hydrogel-embedded VUs were analysed as whole-mounted samples at pre-defined time points (days 1, 3, 5 or/and 7, depending on the experiment). Samples were washed with PBS and fixed with 4 % w/v PFA (30 min, RT). Then, gels were permeabilized with 0.25% v/v Triton X-100 for 20 min, blocked with 5% v/v BSA in PBS for 1 h at room temperature and incubated overnight with Alexa Fluor 647 Phalloidin (Invitrogen, 1:1000), Ulex Europaeus Agglutinin I Rhodamine (UEA I) (Vectors, 1:400) and the following primary antibodies diluted in 1 % w/v BSA: mouse anti-human CD31 (Dako, 1:100); rabbit anti-human fibronectin (FN, Sigma, 1:200); rabbit anti-laminin (Sigma, 1:100); mouse anti-human vimentin (VIM, Santa Cruz, 1:100) and mouse anti-human alpha smooth muscle actin (α -SMA, Abcam, 1:200). Samples were washed with 0.05 % v/v Tween-20 and incubated with the corresponding fluorochrome-conjugated secondary antibodies (Alexa 488 anti-mouse, Alexa 488 anti-rabbit, Alexa 594 anti-mouse and/or Alexa 594 anti-rabbit, Invitrogen, 1:500) and DAPI (1:1000) for 4 h at RT.

Images were acquired using a Leica TCS SP5 AOBS spectral confocal microscope (Leica Microsystems, Germany) or in a high-content screening system equipped with a fully automated widefield/confocal fluorescence microscope (Opera Phenix Plus, Revvity).

2.2. Microfluidic device

Fabrication of the microfluidic chamber and culture-on-chip

The polylactic acid (PLA) core of the chamber was 3D printed using the Fused Deposition Modeling method and the final design was optimized based on a previously reported set-up^[21-23] (Figure S1A, Supporting Information). Briefly, melted PLA layers of 100 μ m height were deposited in precise locations in a layer-by-layer approach to create the object. Then, two fluid dispensing tips of 18 G (Nordson EFD) were inserted into both lateral holes on the sides of the chamber and fixed with epoxy glue. The chamber was then bonded to a glass slide with the same glue and left to dry overnight at room temperature. Pipette tips were used as reservoirs by inserting them into the chamber inlets. For the formation of a perfusable channel in the central reservoir of the microfluidic chamber, an acupuncture needle (300 μ m diameter) was inserted inside the tip inlet and the reservoir was filled with the fibrinogen-thrombin solution for in situ fibrin formation. For studies with VUs, these were added to the hydrogel-precursor solution before loading into the chamber (Figure S1, Supporting Information). After crosslinking, the needle was carefully removed, and the resulting hollow channel was coated

with a fibronectin-collagen solution ^[24]. In some experiments, we used an alternative VoC setup, incorporating two perfusable hollow channels instead of only one (Figure S1, Supporting Information). For endothelialization, ECFC and hIF (10:1 ratio) suspended in EC growth medium were loaded into the channel at C1=1x10⁵, C2=5x10⁵ or C3=10x10⁵ cells per channel. After 2 h, medium was added to the central well and the tip reservoirs. The chips were cultured under static conditions for the first 24 h after channel endothelialization and then transferred to a rocking tilting platform (minimum speed of 4 rpm), which was maintained at 37 °C under a 5% v/v CO₂ humidified atmosphere for 1-2 weeks. Medium (EGM-2MV) inside the channels and central chamber was refreshed daily.

Perfusion assays

The basal fluorescence level of the system was acquired and then fluorescent tracers were added to the microfluidic channels: either soluble dextran (50 µL, 1:100 in PBS, 70 kDa FITC-Dextran, Sigma) or fluorescent microbeads (2 µm size, 1:1000 in PBS, FluoSpheres™ carboxylate-modified microspheres, ThermoFisher Scientific). ECFC were labeled with Agglutinin I Rhodamine (UEA I) (Vectors, 1:400) for analysis of FITC-Dextran or with mouse anti-human CD31 (Dako, 1:100) for analysis of fluorescent beads. Both fluorescent tracers were added to the inlet of the device which was tilted to induce gravity-driven flow to the outlet. After 5 min of perfusion, the device was imaged in a Leica TCS SP5 AOBS spectral confocal microscope (Leica Microsystems, Germany) or in a high-content screening system equipped with a fully automated widefield/confocal fluorescence microscope (Opera Phenix Plus, Revvity).

Gene expression and immunofluorescence analysis

For gene expression analyses, the VUs loaded fibrin hydrogel were removed from the central chamber with a spoon and incubated with 3 mg/mL nattokinase solution for 20 min at 37 °C for fibrin degradation and VUs recovery. Subsequently, RNA extraction, cDNA production and qRT-PCR proceeded as described above for the target genes *PECAM1*, *CDH5*, *LMNA*, *FLT1* (Hs.PT.58.40906831) and *MMP2* (Hs.PT.58.39114006) (Applied Biosystems and Integrated DNA Technologies). Samples were run in triplicates in the ABI Prism 7000 Sequence Detection System under the following conditions: 95 °C for 20 s, followed by 40 cycles at 95 °C for 3 s and 60 °C for 30 s. The expression value for each target gene was normalized to GAPDH value for all the samples.

For immunofluorescence analysis, the whole 3D construct was processed *in situ* as described above for hydrogel-embedded samples and imaged using a Leica TCS SP5 AOBS spectral

confocal microscope (Leica Microsystems, Germany) and the Opera Phenix® Plus High-Content Screening System (Revvity).

2.3. Organoid culture

Crypt Isolation and culture of intestinal organoids (IOs)

Surplus C57bl/6 mice were used for the crypt isolation procedure using a previously published protocol [25, 26]. Briefly, a distal segment of the intestine was harvested, separated from the cecum, and flushed with cold PBS to remove stool. Then, the intestine was opened longitudinally with a scissor and internal mucosa was scraped to remove villi. The tissue was washed in cold PBS, chopped with a surgical blade into 2 to 4 cm pieces and incubated in 500 mM EDTA (Sigma) for 1 h on ice. After incubation, the tissue was resuspended in cold PBS and had-shaken (2 to 3 cycles per second) to dissociate epithelium from the basement membrane and further remove the villi from the crypts. The suspension was transferred to a 70 µm filter and centrifuged for 10 min at 150 g at 4 °C. The pellet was resuspended in PBS and crypts counted in 10 µL drop using a petri dish. A solution of 12,500 crypts/mL of Matrigel (Corning) was prepared and 40 µL domes were seeded in 24 well plates. After 30 min at 37 °C, Intestinal Organoids Medium was added to the wells (Advanced DMEM/F12 with Glutamax and HEPES (Gibco), 1x B27 (Gibco), 1x N2 (Gibco), 1.25 mM N-acetylcysteine (20 mg/mL in H₂O, Sigma), 10% v/v R-spondin (conditioned medium), 10% v/v Noggin (conditioned medium), 100 µg/mL v/v Primocin (InvivoGen), and 50 ng/mL EGF (PeproTech). Medium was changed every 2 days and IOs were passaged every week. To passage or collect IOs for other experiments, 1 mL of ice-cold PBS was added to each well and domes were mechanically dissociated by pipetting up and down vigorously. The suspension was collected to a 15 mL tube and centrifuged for 10 min at 1200 rpm, 4 °C. Supernatant was carefully discarded, and the pellet was resuspended in the appropriate volume of IO medium. Passage ratio was usually 1-to-3 domes. For hydrogel-embedding, IOs alone or combined with VUs were added to Matrigel or to fibrin-based hydrogels. Three types of fibrin-based hydrogels were tested: 1) Fib (non-supplemented fibrin: 1.5 mg/mL fibrinogen with 25 µg/mL aprotinin), 2) Fib-LEC (fibrin supplemented with 2 mg/mL of LEC, laminin-entactin complex, Corning), and Fib-MG (fibrin supplemented with 10 % v/v Matrigel™). To trigger polymerization of fibrin hydrogels, thrombin (2 U/mL) was added at 4:5 v/v ratio.

Oscillation rheometry analysis

The rheological properties of the hydrogels were analyzed by oscillatory shear rheometry (Kinexus Pro rheometer, Malvern) using a cone-on-plate (0.5°/40 mm) geometry. Each gel-precursor solution was prepared and immediately poured on the plate for analysis. A solvent

trap filled with water was used to minimize sample drying. The evolution of the shear moduli, G' (storage, elastic component) and G'' (loss, viscous component), was recorded at 37°C as a function of time for 30 min. A frequency of 0.5 Hz and a strain of 1% were applied to maintain the linear viscoelastic regime. Three replicates were used for each condition.

Immunofluorescence analysis

Samples were processed and imaged as described above but using the following additional antibodies: mouse anti-human β -catenin (β -CAT, BD Biosciences, 1:100); rabbit anti-E-cadherin (E-CAD, Cell signaling, 1:100); rabbit anti-Ki-67 (Abcam, 1:100), rabbit anti-lysozyme (Invitrogen, 1:100).

2.4. Image analysis

Images were analysed using ImageJ 1.50i software and Harmony high-content analysis software 4.8 was used for 3D images reconstructions generation. An image analysis tool developed by Ivanov et al. [27] for Fiji/ImageJ software was adapted to analyze VUs morphology within the molds, using brightfield images. The VUs area was automatically determined, and average diameter was calculated assuming a perfect circle. Sprouting from VUs embedded in fibrin was quantified and morphologically evaluated using the image analysis tools “CLIJ” [28] and “Angiogenesis” [29], for FIJI/ImageJ software (Figure S2B, Supporting Information). Briefly, two Gaussian blur filters with different kernels were applied to a CD31 staining image with “Difference of Gaussians” function of CLIJ, and the two resulting images subtracted to each other, removing background and isolating the sprouts in a new image which was then merged with the corresponding DNA staining image. Thereafter, “Sprout Morphology” function of “Angiogenesis” plug-in was able to detect spheroids, sprouts that were originated from those spheroids, and cell nuclei in each sprout. Different parameters for each step were screened and selected based on sprout and VUs detection efficiency and accuracy.

Sprouting from both cropped sides of the endothelized channel of the microfluidic device was quantified using the image analysis tool “Angiogenesis analyzer” for FIJI/ImageJ software [30]. Briefly, a Median filter followed by a Gaussian blur filter was applied to the cropped right and left sides images acquired from a randomized central area of the channel. Total sprouts number was calculated using the sum of segments and branches detected by the tool.

2.5. Statistical analysis

All the statistical analyses were performed using GraphPad Prism 8.3.0 software. For the data represented in the graphs depicted in Figure 1 and Figure 2, 2-way ANOVA and Bonferroni's

multiple comparisons test were used for the analysis. D'Agostino & Pearson normality test was applied for the graphs of Figures 3E and 3H and the data was analyzed using one-way ANOVA Tukey's multiple comparisons test. All data were analyzed using the non-parametric Mann-Whitney test using a 99% confidence interval and statistically significant differences are marked with * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), or **** ($p < 0.0001$).

3. Results

3.1. VUs generate lumenized microvascular networks inside fibrin

VUs composed by hIF and ECFC combined at cell ratios of 5:1 (10000 hIF + 2000 ECFC) and 1:1 (6000 hIF + 6000 ECFC) were produced and cultured for up to 1 week in non-adhesive agarose microwells (Figure 1A). The overall metabolic activity (Figure 1B) significantly decreased after 5 and 7 days of culture, independently of the cell ratios. This in line with our prior research using dermal fibroblasts or mesenchymal stem cells as supportive stromal cells [9, 10] and is likely associated with the gradual transition from a more active to a nearly quiescent phenotype during spheroid culture. In turn, the cell ratio influenced the VUs size, the ECFC organization, and the global expression of endothelial markers and ECM proteins. VUs with higher proportion of hIF (5:1) presented a smaller size than the 1:1 VUs (Figure 1B), possibly due to the tissue-contraction potential of fibroblasts and their ability to produce large amounts of ECM (Figure 1C, S3 and S4, Supporting Information), which acts as a “glue” contributing to the higher densification and contraction of VUs. Along the time, in both ratios, some of the ECFC (CD31⁺) segregated into the VUs' surface, while others formed clusters at the core, which developed into aligned endothelial structures in the 5:1 VUs (Figure 1C). In VUs with lower proportion of hIF (1:1), some ECFC were gradually excluded from the VUs, as shown by the presence of released CD31⁺ cell clusters (Figure 1C). This was accompanied by a decrease in the number of CD31⁺ cells at the VUs' outer layer from day 1 to day 7 (Figure 1D and S4, Supporting Information). This is possibly due to insufficient stromal support, as previously observed [9, 10]. After 7 days, abundant amounts of ECM components (collagen type I and IV, laminin) were present in VUs, forming a well-defined layer on the surface and exhibiting a more random organization in the interior (Figure 1C). The mRNA expression levels of endothelial and ECM markers (Figure 1E) aligned with the immunofluorescence results. At day 1, the 1:1 VUs (with 3 times more ECFC than 1:5 VUs) showed higher expression of the endothelial markers *PECAM1* (CD31), *CDH5* (VE-cadherin), *KDR* (VEGF receptor 1), *FLT1* (VEGF receptor 1 and the basement membrane (BM) marker *LMNA*). From day 1 to day 7, the expression levels in 1:1 VUs decreased significantly, probably reflecting the loss of ECFC along the culture. In contrast, the expression of *PECAM*, *KDR*, and *FLT1* in 5:1 VUs increased from day 1 to 7, which may reflect the higher degree of ECFC organization and formation of

primitive vessel-like structures at the VUs core. In both ratios, there was an increased expression of *Col1a1* from day 1 to day 7, which likely reflects sustained ECM production and accumulation inside VUs.

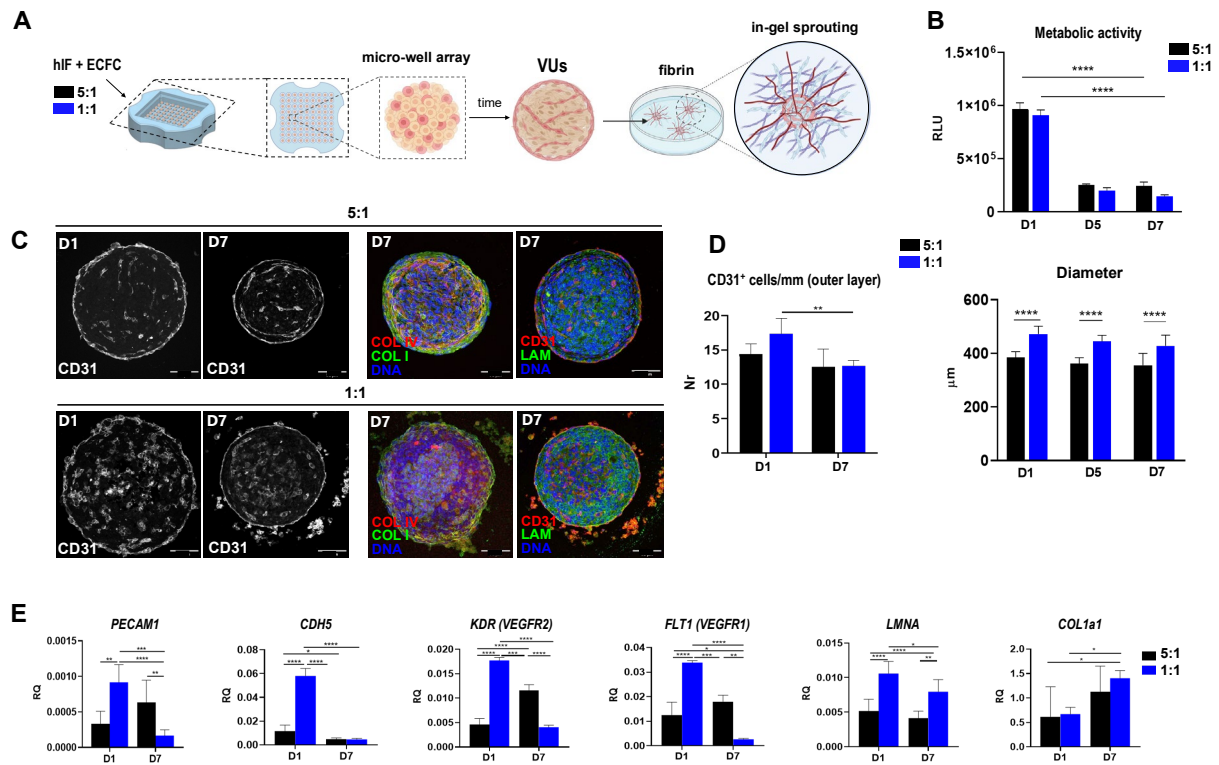


Figure 1. Endothelial cells segregate and organize at the outer layer and at the core of VUs. **A)** Graphical representation of VUs production using agarose microwell-molds and subsequent embedding in fibrin hydrogel. Created with BioRender.com. **B)** Metabolic activity and diameter of VUs with different hIF-to-ECFC ratios (5:1 and 1:1) after 1 to 7 days of culture (n=3 independent experiments, 2 technical replicates each). **C)** Immunofluorescence images showing the organization of ECFC (CD31⁺ cells) in VUs (5:1 and 1:1) after 1 and 7 days of culture and ECM production at day 7 (scale bars: 75 µm). **D)** Quantification of CD31⁺ cells on the outer layer of VUs (5:1 and 1:1) at day 1 and 7. **E)** mRNA expression (qPCR analysis) of *PECAM1* (encodes for CD31), *CDH5* (encodes for VE-cadherin), *KDR* (encodes for VEGFR2), *FLT1* (encodes for VEGFR1), *LMNA* (encodes for laminin) and *Col1a1* (encodes for collagen type I). In all graphs, statistically significant differences are marked with * (p < 0.05), ** (p < 0.01), *** (p < 0.001), or **** (p < 0.0001).

After characterization, VUs were collected after 1 day of spheroid culture, when they showed higher metabolic activity and higher ECFC amount, embedded in fibrin and cultured for an additional week. The hydrogel provided a favorable 3D environment for angiogenic sprouting, leading to the rapid formation of multiple VUs-derived capillary-like structures. As the culture progressed over 7 days, the number of sprouts decreased, as they merged into larger diameter structures, and their length increased (Figures 2A and 2B). As the 1:1 VUs showed enhanced levels of active sprouting (shortly after embedding) and lengthier sprouts (after 7 days of

culture) they were selected for the subsequent studies. The VUs-derived capillaries were surrounded by a laminin-rich BM-like layer and presented distinct lumens (Figure 2C). The hIF (VIM⁺ cells) were also observed to migrate out of the VUs and invade the surrounding hydrogel, contributing to the production of ECM in the external space and to the stabilization of the formed microvascular structures (Figure 2D). Notably, α -SMA⁺ cells were found close to vWF⁺ microvessels, suggesting that some of the hIF committed to pericyte-like mural cells (Figure S5, Supporting Information).

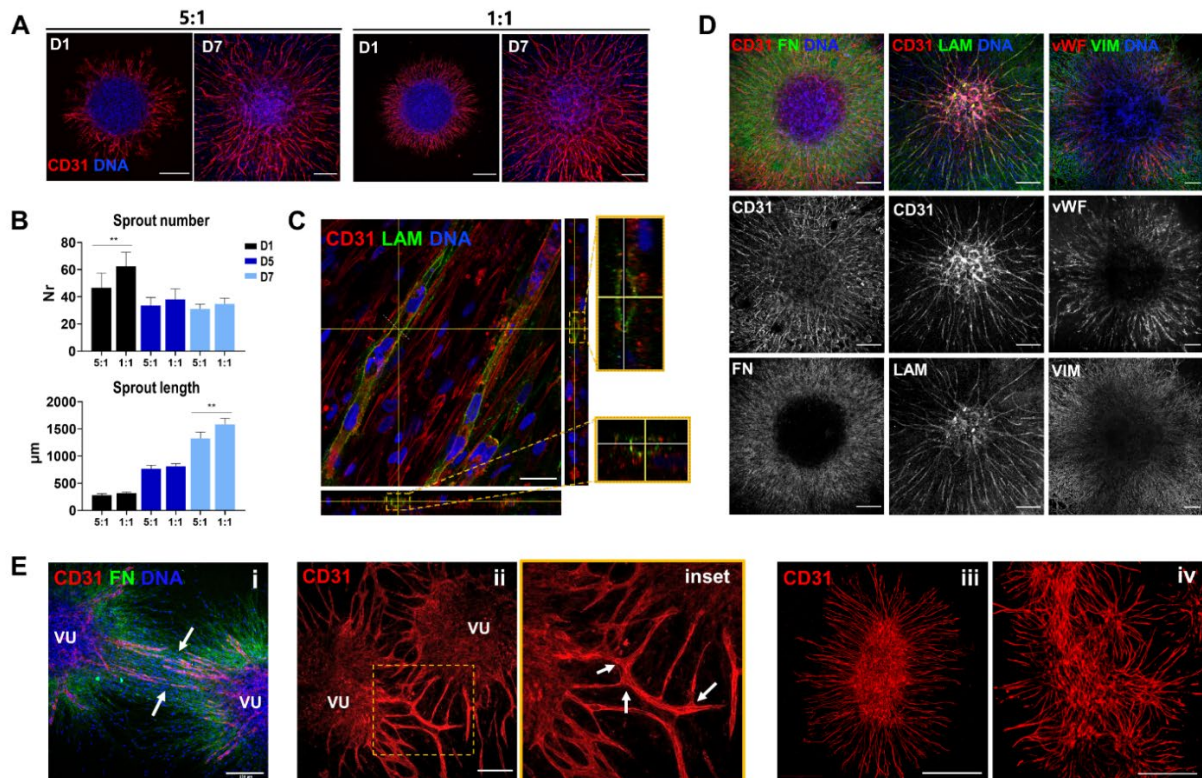


Figure 2. VUs form extensive microvascular 3D networks when embedded in fibrin. **A)** Immunofluorescence images showing angiogenic sprouting from VUs in fibrin (5:1 and 1:1) at days 1 and 7 post-embedding (red: CD31, blue: nuclei, scale bars: 250 μ m). **B)** Average number and length of VUs-derived capillaries (5:1 and 1:1) at days 1, 5, and 7 post-embedding. **C)** Higher magnification image showing a laminin-rich BM-like layer enveloping the VUs-derived capillaries (green: laminin, red: CD31, blue: DNA, scale bar: 50 μ m) and the presence of lumens (orthogonal views). **D)** Immunofluorescence images showing VUs-derived capillaries (CD31⁺ cells), outward migration of hIF (VIM⁺ cells), and deposition of ECM proteins (LAM and FN) in the fibrin hydrogel surrounding 1:1 VUs after 7 days in culture (scale bars: 250 μ m). **E)** Immunofluorescence images showing: i) the ability of VUs-derived capillaries (1:1) to grow towards each other and ii) inosculate; and iii) the ability of VUs to fuse with other VUs and iv) form extensive microvascular networks (red: CD31, green: FN, blue: DNA). Scale bars: 250 μ m (i), 200 μ m (ii); 1 mm (iii-iv). In all graphs, statistically significant differences are marked with * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), or **** ($p < 0.0001$).

Remarkably, capillaries originating from neighboring VUs showed the ability to grow towards each other (Figure 2E-i) and merge (Figure 2E-ii). This inosculation potential, along with the ability of VUs to fuse with other VUs at the core level (Figure 2E-iii), ultimately led to the

formation of extensive microvascular networks (Figure 2E-iv) with elongated capillaries spanning up to 5 mm in length after 1 week of culture. These structures remain stable for at least 2 weeks in culture (data not shown). Collectively, these findings showcase the suitability of VUs as building blocks for the bottom-up generation of robust microvascular 3D networks.

3.2. Establishment and perfusion of a biomimetic fibrin-based VoC

To further characterize the VUs-derived capillaries and microvascular networks, we designed a customized microfluidic device (vessel-on-chip, VoC), incorporating a hollow channel within the fibrin hydrogel, all contained within a perfusable PLA holder (Figure 3A). The hydrogel precursor solution was introduced into the central chamber of VoC over a needle that was removed after polymerization, forming a hollow channel (with an average diameter of ~ 300 μm). This arteriole-like microfluidic channel was perfused with a blue dye using a peristaltic pump, which validated its flexibility, structural integrity, and ability to accommodate flow rates within the range of 1 to 10 $\mu\text{L}/\text{min}$ without bursting (Supporting Information, videos). To ensure successful endothelialization, the hollow channel was pre-coated with ECM proteins prior to cell seeding (Figure 3B). Notably, the entire structure, i.e., the hydrogel matrix with the embedded channel, with or without cells, could be readily retrieved from the PLA chamber using a spatula, without compromising its structural integrity (Figure 3C i-v). Additionally, the fibrin hydrogel could be enzymatically degraded under mild conditions yielding a self-standing endothelialized vessel-like structure (Figure 3C vi-vii and Figure S6, Supporting Information), which greatly facilitated downstream analysis.

We tested various cell densities: C1 (1×10^5 cells), C2 (5×10^5 cells), and C3 (10×10^5 cells), to promote the endothelialization of the hollow channel (Figure S7, Supporting information). Our findings revealed that the cell density had a significant impact on the performance and organization of the resulting endothelial layer. While the lower cell-seeding density (C1) resulted in a stable endothelial monolayer (Figure 3D and 3E), higher cell densities (C2, C3) led to the formation of a pro-angiogenic endothelium, from which lumenized endothelial sprouts emerged, extending into the hydrogel bulk (Figures 3D and 3E). The hIF also migrated from the microchannel into the fibrin, contributing to the formation of stromal tissue within the hydrogel (Figure 3F).

Notably, the VoC channel-derived endothelial sprouts presented lumens up to 110 μm in diameter (Figure 3G), with no significant differences between the C2 and C3 conditions in terms of sprout number and lumen diameter (Figures 3E and 3H). Based on these findings, the intermediate condition (C2) was selected for further studies.

By perfusing the VoC microfluidic channel with FITC-Dextran (70 kDa), we provided evidence of “flow-through”, with no apparent leakage, and of “flow-in” into the angiogenic sprouts (Figure 3I). The VoC channel was also perfused with fluorescent microbeads (2 μ m), which infiltrated the endothelial sprouts and accumulated at their open-ended branches, within the hydrogel matrix (Figure 3J). For some of the studies with VUs (section 3.3), we used an alternative VoC setup, incorporating two perfusable hollow channels instead of only one. Overall, this customized VoC, composed only of fibrin and cells, provided an ideal biomimetic platform with no geometrical constraints to microvessel development, as envisaged.

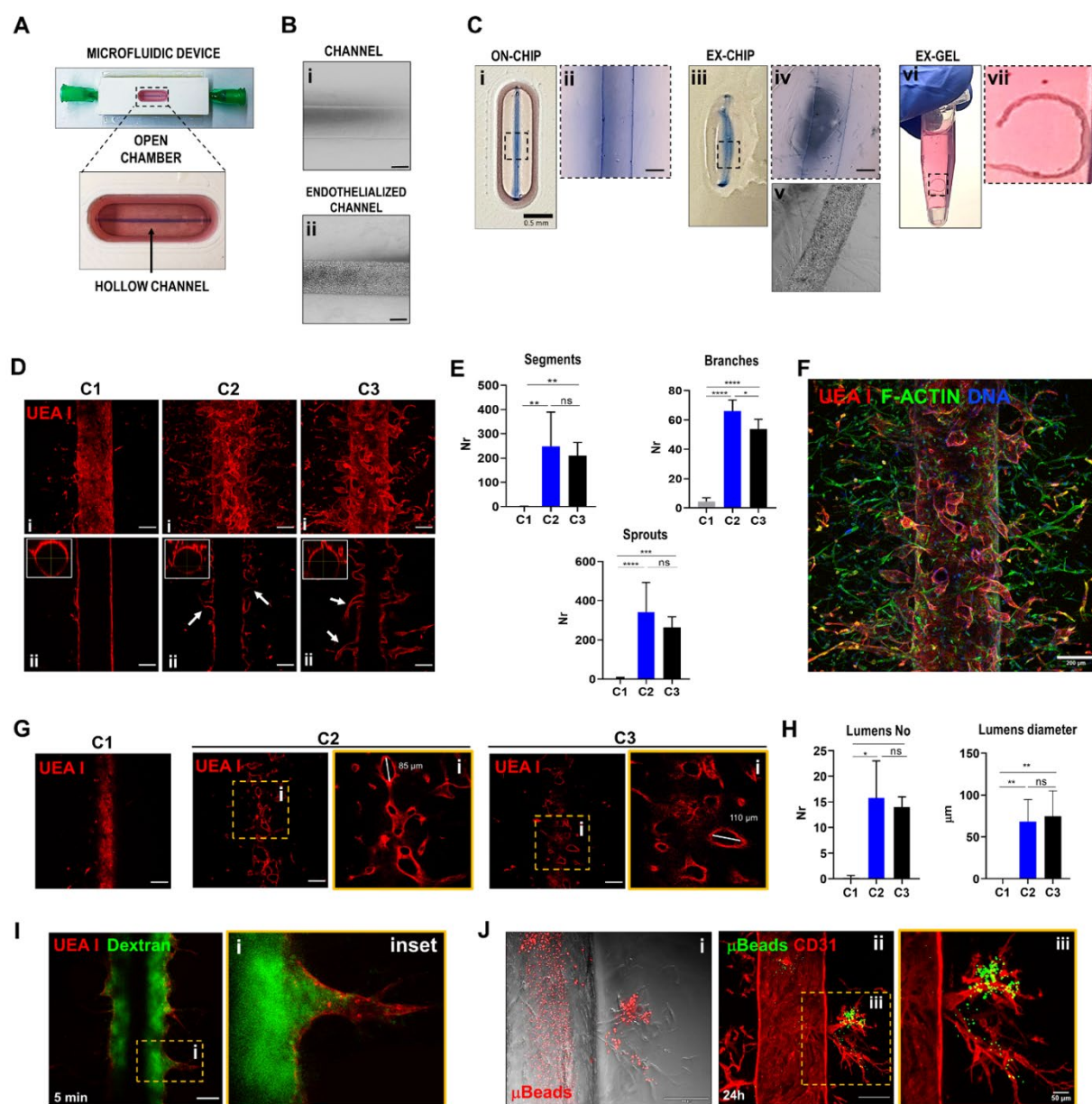


Figure 3. The designed fibrin-based microfluidic device offers a biomimetic VoC. **A)** Top view of the microfluidic device showing the hollow channel perfused by blue dye. **B)** Phase-contrast images of the hollow channel (i) before and (ii) after endothelialization. Scale bars: 200 μm . **C)** i) image of the chamber perfused with blue dye (ii: magnification, scale bar: 200 μm); iii) image of the fibrin hydrogel removed from the chamber (magnifications showing iv-bare and v-endothelialized channels, scale bar: 200 μm); vi) image of endothelialized channel after enzymatic degradation of fibrin (vii: magnification). **D)** Representative immunofluorescence images of VoC loaded with different cell densities: 1×10^5 (C1), 5×10^5 (C2) or 10×10^5 (C3) cells (red, UEA I), (i-longitudinal and ii-transversal stacks showing the channel lumen and the lumenized endothelial sprouts (white arrows), scale bars: 200 μm . **E)** Quantification of the total number of segments, branches, and sprouts in representative areas of the VoC at different cell densities ($n=3$ independent experiments, 2 channel areas per experiment). **F)** Immunofluorescence image showing the different cell types (ECFC and hIF, at C3) sprouting from the channel (red: UEA I, green: F-actin, blue: DNA, scale bar: 200 μm). **G)** Top stack view of the channel with different cell densities (C1, C2 and C3) showing the absence of endothelial sprouts in C1, and the presence of lumenized endothelial sprouts in C2 and C3 (red: UEA I, scale bars: 200 μm). **H)** Quantification of lumens number and diameter ($n=3$ independent experiments, 2 channel areas per experiment). **I)** Immunofluorescence image showing a transversal stack of the VoC (red: UEA I) 5 min after perfusion with 70 kDa Dextran-FITC (green), scale bars: 200 μm (i: magnification). **J)** Perfusion of VoC with fluorescent microbeads for 5 min: i) phase-contrast composite image obtained immediately after perfusion (red: beads), ii) immunofluorescence image obtained 24 h after perfusion (red: CD31, green: beads), scale bars: 200 μm , iii) higher magnification image, scale bar: 50 μm . In all graphs, statistically significant differences are marked with * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), or **** ($p < 0.0001$).

3.3. The VUs-derived capillaries inosculate with VoC channels and form extensive microvessel networks

To further demonstrate the ability of VUs to generate extensive microvascular networks these were loaded into the VoC. The central chamber was filled with fibrin precursor solution and VUs were introduced close to (1-channel VoC) or between channels (2-channels VoC, Figure 4A), prior to complete polymerization. As shown in Figure 4B, the endothelial sprouts emerging from the VoC channel were unable to bridge the 2 mm gap between them and form connections with each other, even after 6 days of culture. In contrast, when VUs were also incorporated into the VoC, they rapidly formed capillaries that spanned the 2 channels, creating a microvascular 3D network. Figure 4C depicts more detailed images of the system, showing VUs next to a VoC channel immediately after fibrin-embedding (day 0, 4C-i) and the formation of channel-derived and VUs-derived capillaries over time (day 5, 4C-ii). The length of the VUs-derived capillaries progressively increased, and the interactions between adjacent VUs contributed to the formation of a capillary network (Figure 4C-ii and Figure S8, Supporting Information). At day 5 there was already inosculature between capillaries from neighbouring VUs (VU-VU, Figure 4C-iii, dashed arrows), as well as between VUs-derived capillaries and the VoC channel (VU-VoC, Figure 4C-iii, solid arrows). This facilitated the expansion of the microvascular network throughout the entire device. Figure 4C-iii also shows the presence of fibroblasts (CD31⁻ cells stained for f-actin, in green) that migrated from VUs, and formed a stromal network within the bulk fibrin hydrogel, around the VUs and the VoC channels. The stromal-embedded microvasculature became progressively more stable, transitioning from an 'active' pro-angiogenic state to a more mature vascular phenotype. This was corroborated by

a decrease of the mRNA expression levels of endothelial markers (*PECAM1*, *CDH5*, *FLT1*), BM markers (*LMNA*), and matrix-remodeling markers (*MMP2*) decreased from day 1 to day 7 (Figure 4D)^[31, 32].

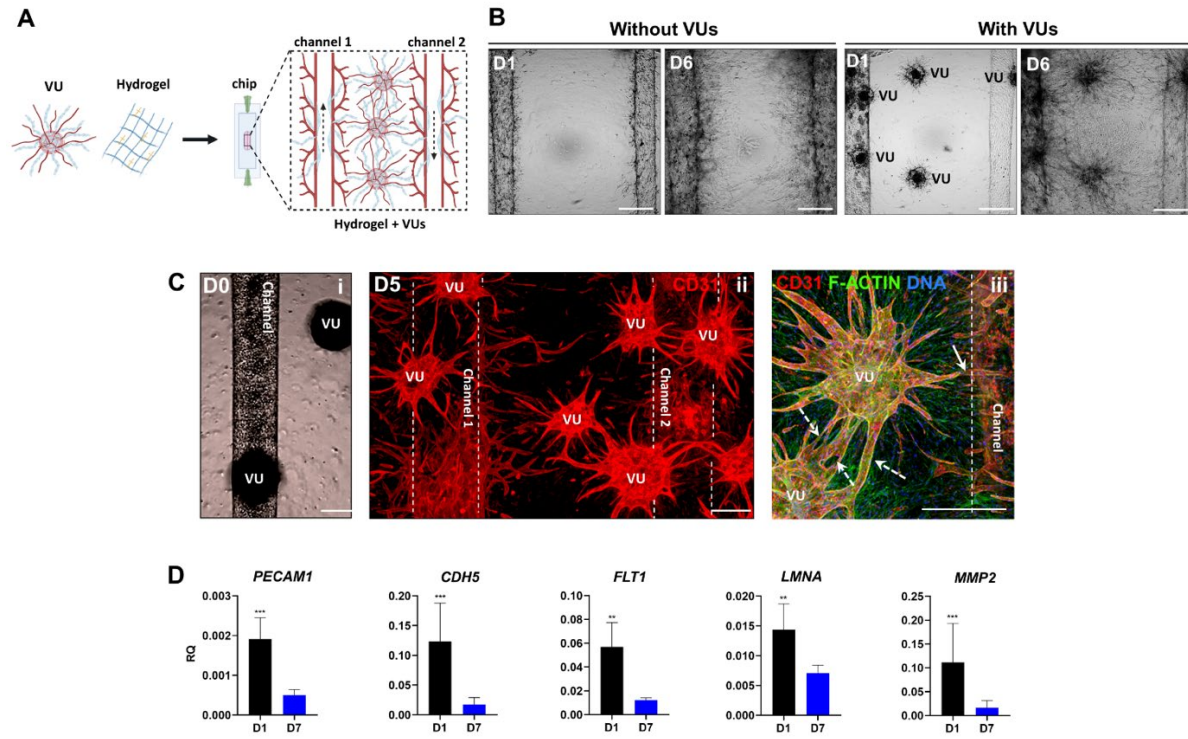


Figure 4. VUs bridge the VoC channels and expand the microvascular network to the whole device. **A)** Graphical representation of the experimental setting of VUs assembly in the microfluidic device. Created with BioRender.com. **B)** Phase contrast images of the 2-channel VoC design without and with VUs at day 1 and 6 (scale bars: 500 μ m). **C)** Phase-contrast (i) and fluorescent images (ii, iii) of VUs integrated in the chip at days 0 and 5. (iii) higher magnification image (red: CD31, green: F-actin, blue: DNA, white dashed line: channel, scale bars: 200 μ m) showing interconnections between VUs-derived capillaries (dashed arrows) and between VUs-derived capillaries and the channel (solid arrow). Fibroblasts correspond to the f-actin stained CD31⁺ cells. **D)** mRNA expression of *PECAM1* (encodes for CD31), *CDH5* (encodes for VE-cadherin) and *FLT1* (encodes for VEGFR1), *LMNA* (encodes for laminin) and *MMP2* (encodes for matrix metalloproteinase 2) at days 1 and 7. In all graphs, statistically significant differences are marked with ** (p < 0.01) or *** (p < 0.001).

3.4. The VUs-derived microvascular network is perfusable

Figure 5A depicts the 3D volume visualization by Thermal Color LUT image of UEA-I⁺ staining (ECFC cells), showcasing the close-distance positioning of a VU in relation to the VoC channel (Figure 5A-i) and the interconnection between both structures (Figure 5A-ii). While there was no significant difference in the average distances between VUs (VU-VU) and between VUs and the channel (VU-VoC) (Figure 5B-i), the VU-VU interconnections were more frequent than the VU-VoC (Figure 5B-ii). This was somewhat expected since there is only one channel but multiple VUs. Additionally, quantitative data demonstrated that the diameters of the VUs-derived microvessels had a homogenous size distribution, ranging from 10 to 40 μ m, whereas

the VoC-derived microvessels were significantly larger and more heterogeneous, ranging from 30 to 110 μm (Figure 5B-iii).

To demonstrate the perfusability of the VUs-derived capillaries, fluorescent microbeads were injected into the VoC channel. As shown in Figure 5C-i, in regions where VUs inosculated with the VoC channel, the flow was able to pass through the VoC into the VUs-derived capillaries, which also became perfused (Figure 5C-ii), as evidenced by the presence of fluorescent microbeads flowing along them (Figure 5C-iii).

Figure 5D shows a robust VUs-derived microvessel network surrounding the VoC channel, along with 3D reconstruction images of selected regions (Figure 5E i-iii) that clearly demonstrate the presence of fluorescent microbeads inside the lumen of VUs-derived capillaries. It also highlights the presence of the fibroblastic stromal network, surrounding both the VUs-derived capillaries and the VoC channel (Figure 5E iv-vi). Collectively, these findings confirm the effective inosculature between the VUs and VoC channel, demonstrating the perfusability of the generated stromal-embedded microvascular 3D network.

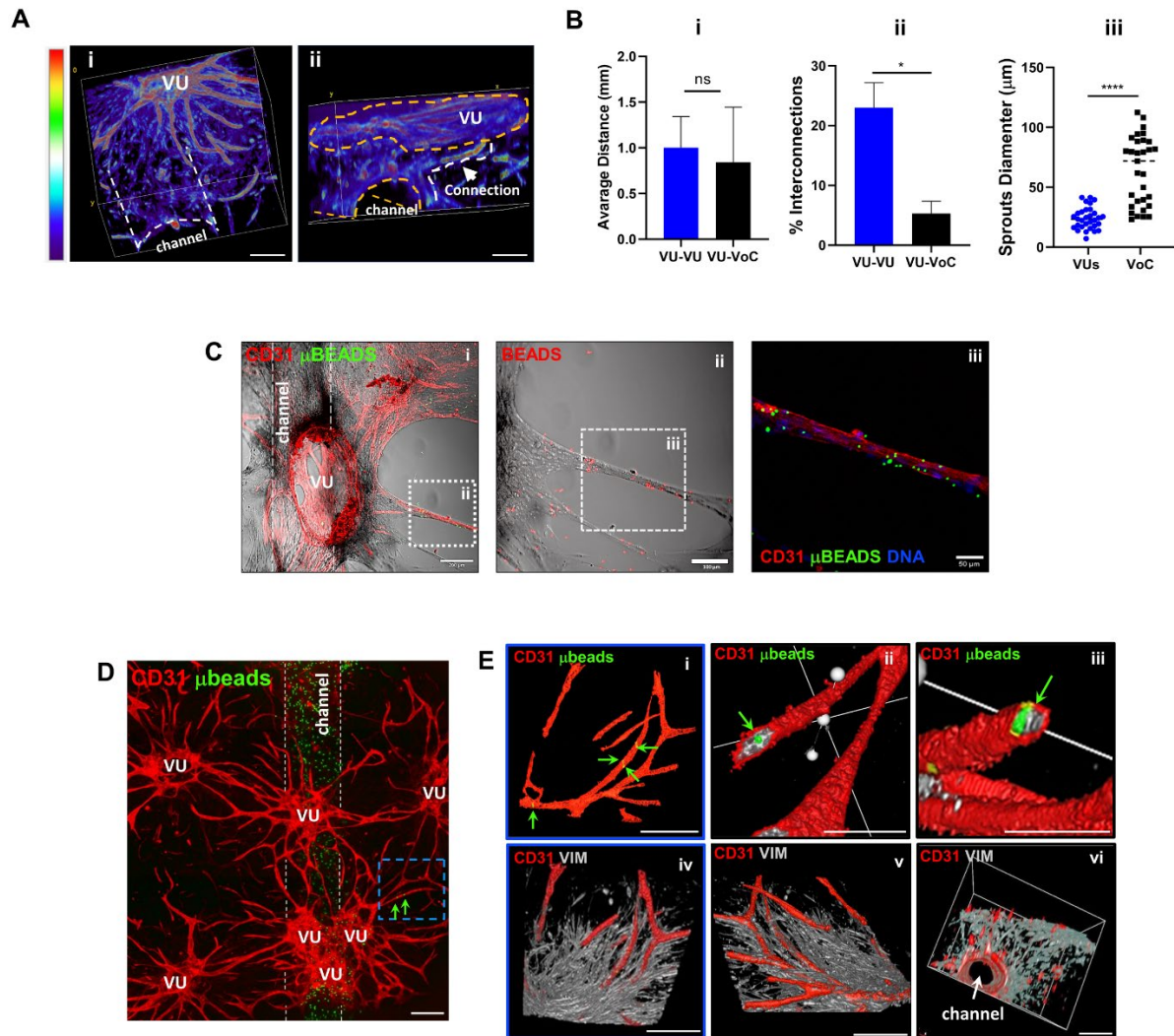


Figure 5. Inosculation and perfusion of VUs-derived capillaries in the VoC. **A)** Representative Thermal Color LUT 3D reconstructions of UEA-I⁺ staining showing the (i) relative position and (ii) interconnection zones between VU and VoC (dark blue: low signal intensity, red: high signal intensity, scale bars: 200 μ m). **B)** Quantification of: (i) average distance between VUs (VU-VU) and between VUs and the VoC (VU-VoC), (ii) percentage of VU-VU and VU-VoC interconnections, and (iii) average diameter of VUs-derived and channel-derived microvessels. In all graphs, statistically significant differences are marked with ** ($p < 0.01$) or *** ($p < 0.001$). (iv-v). **C)** Composite image of VUs connected with the VoC after 7 days of culture (red: CD31; green: fluorobeads, scale bar: 200 μ m). (i) higher magnification image of the yellow dashed region (red: fluorobeads, scale bar: 100 μ m). (ii) higher magnification image of red dashed region (red: CD31, green: fluorobeads; blue: nuclei, scale bar: 50 μ m). **D)** Confocal image showing VoC perfusion with fluorescent beads (2 μ m) and perfusate infiltration into VUs-derived microvessels at day 7 (dashed lines: VoC; green arrows: infiltration of fluorescent beads inside VUs-derived microvessels; red: UEA I, green: fluorescent beads; scale bar: 200 μ m). **E)** (i-iv and v) 3D reconstructions showing a higher magnification of Figure 4F blue dashed zone and stromal network supporting VUs-derived microvessels (red: CD31, gray: vimentin, green: fluorescent beads; green arrows: fluorescent beads, scale bar: 200 μ m). (ii and iii) 3D reconstruction from confocal z-stacks using clipping planes to reveal the presence of fluorescent beads located inside the lumen of VUs-derived microvessels (red: CD31, green: fluorescent beads; green arrows: fluorescent beads, scale bar: 200 μ m). (vi) 3D representation of VoC showing a rich stromal compartment surrounding the vascular channel (red: CD31, gray: vimentin; scale bar: 200 μ m).

3.5. VUs promote the formation of a vascularized stroma around IOs in the VoC

As a preliminary proof-of-concept, we assessed the potential of VUs to promote the formation of a vascularized stroma around intestinal organoids. Before co-culturing VUs with IOs in the VoC, we set out to replace Matrigel (MG), which is the standard 3D matrix for IOs culture, by fibrin-based hydrogels. We tested three different fibrin hydrogel formulations: Fib (non-supplemented fibrin, the one used for VUs culture); Fib-MG (fibrin supplemented with MG); and Fib-LEC (fibrin supplemented with LEC: laminin-entactin complex). The rheological characterization (Figure 6A) demonstrated that Fib yielded a softer matrix compared to Matrigel (MG), while Fib-LEC hydrogels exhibited a significant increase in the G' value, reaching levels comparable to MG. Despite the differences in rheological properties, the organoid formation efficiency (% of organoid development from single cysts) was similar between Fib-MG, Fib-LEC and MG (Figure 6B). While Fib hydrogels alone did not support the growth of passaged IOs, the Fib-MG and Fib-LEC hydrogels facilitated the development of rounded cysts into characteristic crypt-like budding structures within 4 days. Over the course of 7 days, these structures underwent further maturation, displaying a growth pattern similar to that observed in MG (Figure S9, Supporting information). As depicted in Figure 6D, the IOs displayed budding structures surrounding the central lumen, similar to the intestinal crypt domain, with polarized cellular organization and expression of the epithelial marker β -catenin (Figure 6C). Moreover, the budding domains were enriched in Ki67+ proliferative cells (Figure 6D and 6D li ii), and in terminally differentiated Paneth cells, as shown by the expression of C-type lysozyme (Figure 6D iii, iv and Figure S10, Supporting information).

We then tested the behaviour of IOs when cultured in fibrin-based hydrogels in the VoC. Passaged organoids were embedded in Fib-MG or Fib-LEC hydrogel precursor solutions and added to the microfluidic device. After polymerization, the hollow channel was endothelialized as previously described. Over time, in both hydrogels, IOs were able to grow and develop into more complex structures (Figure 6E), interacting with the channel-derived endothelial sprouts close to the VoC channel walls.

Finally, to extend the microvascular network into the hydrogel bulk, passaged IOs were combined with VUs within the VoC (Figure 6F). Both types of structures were dispersed in the fibrin hydrogels prior to polymerization, and 3 days later, the VUs already presented angiogenic sprouts, similar to those observed in non-supplemented Fib hydrogels, while the IOs continued to grow and differentiate (Figure 6G). Over time, the VUs-derived capillaries progressively reached and enveloped the IOs (Figure 6G and 6H), both proximal and distal to the VoC channel, expanding the microvascular network to multiple organoids. At the same time, the

fibroblasts formed multicellular networks around the IOs, providing additional stromal support to the system and enhancing its physiological relevance.

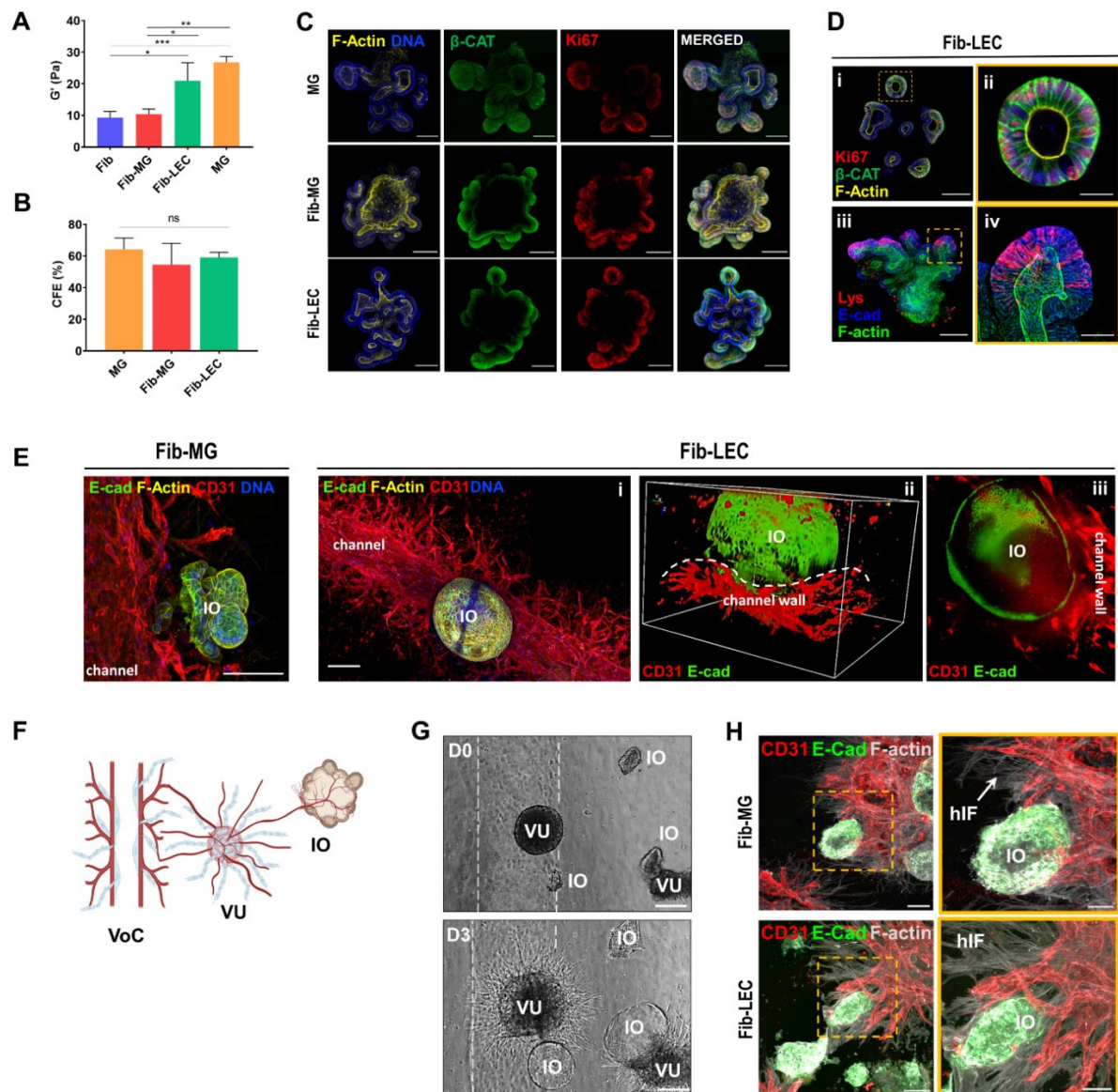


Figure 6. VUs promote the formation of a vascularized stroma around IOs in the VoC. **A)** Single frequency sweep analysis of G' of Fibrin, Fibrin-Matrigel, Fibrin-LEC and Matrigel (MG) during gelation ($n=3$). **B)** Colony-forming efficiency of intestinal crypts embedded in different hydrogels. **C)** Representative immunofluorescence images of IOs cultured for 7 days in Fib-MG, Fib-LEC or MG showing similar morphology (yellow: F-actin, green: β -catenin, red: Ki67, blue: nuclei). Scale bars:100 μ m. **D)** (i) Representative immunofluorescence images of IOs cultured in Fib-LEC showing proliferative Ki67+ cells (yellow: F-actin, green: β -catenin, red: Ki67, blue: nuclei). Scale bar:100 μ m. (ii) Higher magnification image of the yellow dashed region. Scale bar: 30 μ m. (iii) Expression of the phenotypical intestinal marker lysozyme (green: F-actin, blue: E-cadherin, red: lysozyme). Scale bar:100 μ m. (iv) Higher magnification image of the yellow dashed region. Scale bar: 30 μ m. **E)** IOs embedded in Fib-MG or Fib-LEC hydrogels integrated into the microfluidic chamber and cultured in close contact with the VoC (yellow: F-actin, green: E-cadherin, red: CD31, blue: DNA). Scale bars: 100 μ m. **F)** Graphical representation of the experimental approach where VUs promote IOs vascularization. **G)** Phase-contrast images of VUs and IO integrated into the microfluidic chamber and in close contact with the VoC at days 0 and 3. Scale bar: 100 μ m. **H)** Fluorescent images depicting VUs-derived endothelial sprouts and rich stromal network in close proximity to IOs after 3 days of culture in Fib-MG or Fib-LEC (F-actin: grey, red: CD31, green: E-cadherin, blue: DNA). Scale bars: 100 μ m (inset images: 50 μ m).

4. Discussion

The timely establishment of perfusable microvascular networks within engineered tissues and organs is essential to ensure their viability and function upon implantation. These microvascular networks should ideally develop *in situ*, adapting to the local microenvironment, and rapidly inosculate with host microvessels to extend blood flow throughout the entire 3D construct [4, 33, 34]. In this study, we present an innovative vascularization strategy based on the use of Vascular Units (VUs) as building blocks to generate stroma-embedded perfusable microvascular networks from the bottom-up. Thanks to their versatility and micro-sized nature, VUs can be directly injected into a lesion site or easily integrated into different types of 3D scaffolds before implantation.

In this study, we selected the small intestine as the target organ given the urgent need for tissue engineering solutions in intestinal wound healing, and the vital role of a supportive vascularized stroma in epithelial regeneration. Considering that fibroblasts from different tissues exhibit distinct molecular signatures, particularly in terms of ECM composition [35], we produced VUs combining ECFC with organ-specific intestinal fibroblasts to better mimic the vascularized stromal compartment of the small intestine. Several studies have emphasized the key role of stromal cells in the formation of vascularized microtissues, where they modulate EC behavior and assembly into lumenized capillary-like structures [8-10, 14, 36, 37]. Stromal cells may also act as pericytes, contributing to the long-term stability of these structures [20, 37-40]. Here, the presence of intestinal fibroblasts was central for ensuring the cohesiveness and structural integrity of the VUs, which could not be achieved when using ECFC alone (data not shown), as previously demonstrated with other stromal cell types [10]. Moreover, when VUs were embedded in fibrin, fibroblasts co-migrated into the hydrogel, secreting their own ECM. This contributed to the establishment of an optimal 3D microenvironment for vascular morphogenesis, resulting in the generation of robust capillary beds stabilized by interstitial matrix and a basement membrane layer, ultimately forming an ECM-rich vascularized stroma. We demonstrated that the VUs-derived capillaries presented well-defined lumens and were able to inosculate with capillaries from adjacent VUs, forming extensive microvessel 3D networks.

To further characterize the inosculation potential and perfusability of the VUs-derived capillaries, we designed a biomimetic fibrin-based vessel-on-chip (VoC). This microfluidic device imposed no geometrical restriction on vascular growth and supported the formation of hierarchical and interconnected microvessel networks, emulating the physiological behaviour of native vasculature. It featured one or two fibrin-embedded hollow channels with diameter in the range of arterioles/venules, created using an acupuncture needle as the template. While

not novel, this approach was very reproducible and more straightforward compared to sacrificial molding technique ^[14, 41, 42]. It was also versatile, as the channel diameter could be easily adjusted by selecting needles of different sizes. For endothelialization, it was necessary to coat the hollow channel with ECM proteins to facilitate cell attachment, and the co-seeding of hIF along with ECFC in the channel was essential to achieve a cohesive endothelial monolayer. Moreover, the density at which cells were seeded played a pivotal role in determining the angiogenic sprouting potential of the endothelialized channel. Higher cell densities promoted the formation of lumenized endothelial sprouts, and lower densities resulted in a steady endothelial layer, possibly due to insufficient coverage for a complete monolayer soon after seeding. While this has not been studied in detail herein, it is also possible that the effective adhesion ratio of hIF-to-ECFC to the channel varies under the different conditions, which in turn is likely to impact the behavior of ECFC. Moreover, prior research showed the positive impact of higher cell densities on angiogenic sprouting ^[43, 44]. The open-ended endothelial sprouts that emerged from the VoC channel infiltrated the surrounding hydrogel, forming a vascular tree-like structure. They were also perfusable and non-leaky, as demonstrated by flow studies using fluorescent dextran (70 kDa) or fluorescent microbeads (2 μ m). This dynamic feature of neo-vessel formation sets our model apart from other reported VoCs with steady vascular-like channels ^[45-47]. Of note, the fibrin-embedded hollow channels sustained different flow regimens without bursting, and the 2-channel set-up represents a step forward in modelling a closed circulation loop integrating both arteriole-like and venule-like flow^[48]. While not further explored in this study, these are relevant features of the system that will allow exploring cells' response to fluid dynamics in future studies ^[3, 49]. This VoC design offers some advantages over traditional PDMS-based organ-on-chip (OoC) platforms, namely: i) it delivers a biomimetic vascularized 3D construct, i.e., with no additional plastic components apart from the PLA housing; ii) it allows for the culture and recovery of large sample volumes, and iii) it allow the extraction of the entire vascularized 3D construct from the PLA housing without compromising its structural integrity. Overall, this greatly eases handling, monitoring, and downstream analysis.

When VUs were added to the fibrin hydrogel between 2 mm spaced channels within the VoC, the VUs-derived capillaries bridged the channels and contributed to the establishment of a robust microvascular network throughout the entire device. Notably, these microvascular structures were embedded in a stromal microenvironment rich in ECM and fibroblasts, which together supported the growth and stability of the capillary bed. The progressive stabilization of these vascular structures translated into a decrease in the mRNA expression of endothelial activation, angiogenesis, and matrix remodelling markers. These processes are regulated by VEGF/Notch signalling pathways, and vessel differentiation, linked to Notch activation, is

characterized by cell cycle arrest and downregulation of VEGF-regulated endothelial gene expression markers, as observed herein [31, 32].

The VUs-derived capillaries also successfully inosculated with capillaries from other VUs (VU-VU) and with the VoC channel-derived capillaries (VU-VoC). As a result, when the VoC channel was perfused, some of the VUs-derived capillaries also become perfused, as shown by the presence of fluorescent beads (used as tracers) inside their lumens. While the VoC could be continuously perfused, characterizing the perfusability of the VUs-derived networks was challenging due to the non-standard flow properties of the fluorescent microbeads. Indeed, the effective infiltration of the inosculated capillaries by the flowing microbeads was a highly probabilistic event, leading to an underestimation of the system's perfusion capability. Additionally, the use of fluorescent dextran was not ideal, as the presence of the open-ended endothelial sprouts promoted its rapid diffusion throughout the hydrogel matrix, creating imaging artifacts. Despite these limitations, our results clearly demonstrate that VUs are effectively able to functionally inosculate with larger diameter vessels. While requiring *in vivo* validation, this suggests their capability to connect with host vessels upon implantation, become blood-perfused, and form functional microvascular networks *in situ*. Notably, the use of VUs as a (micro)vascularization strategy can be explored to engineer various types of tissues and organs, highlighting their broad applicability in the regenerative medicine field.

The potential of VUs to generate extensive stroma-embedded microvascular networks within a VoC device and 'bridge' perfusable channels also represents a novel and relevant concept in the field of microphysiological systems. Indeed, such an approach could facilitate the establishment of microfluidic loops in VoC models lacking effective connections between two or more vascular channels [50-52]. Another important advantage of VUs is that they also contain fibroblasts, which gradually migrate into the hydrogel compartment, creating an ECM-rich stroma "on-chip". This is crucial for more accurately mimicking the natural environment of tissues/organs and advancing the field of OoC [53].

As a preliminary proof-of-concept, we demonstrated the ability of VUs to promote the formation of a vascularized stroma around intestinal organoids within the VoC. Before combining VUs with IOs, we had to optimize fibrin-based hydrogels for organoid culture, as fibrin alone proved to be unsuitable for IOs development. Fibrin supplemented with Matrigel or laminin-111/entactin complex (LEC) yielded excellent results, while also supporting the angiogenic sprouting potential of the VUs, allowing the successful combination of the different structures in the VoC. This is in accordance with previous findings from other authors showing that IOs cultured in LEC-supplemented fibrin hydrogels present colony formation efficiency and differentiation potential comparable to that of pure MG [54]. Thus, the supplemented fibrin

hydrogels, particularly the Fib-LEC, offer an interesting alternative to Matrigel, as they present a more defined and consistent composition that may enhance the reproducibility of IOs culture [55]. When IOs were added to the supplemented fibrin hydrogels in the VoC they continued to grow, and those located close to the wall of the endothelialized channel became rapidly surrounded by channel-derived capillaries. Therefore, the inclusion of VUs alongside IOs enabled the simultaneous vascularization of multiple organoids within the VoC, even those located distant from the VoC channel, while also forming a fibroblast-derived stromal tissue around them. This VUs-based strategy has the potential to substantially increase the surface contact area of IOs with a vascularized stroma and enable the culture of multiple organoids per chip, in contrast to other VoC platforms that have compartmentalized channels and space constraints [37, 39, 51]. Our results are promising, but future studies should focus on analyzing IOs functionality in the presence and absence of a vascularized stroma under perfusion.

Overall, the small size and high angiogenic sprouting potential of VUs establish them as versatile tools for engineering perfusable microvascular networks in various contexts. They can be: i) integrated into in vitro models to enhance their biomimicry, including in VoC platforms as illustrated herein; ii) combined with a vehicle and used in an injectable form for minimally invasive therapeutic vascularization [9]; or iii) combined with scaffolds [10] or with bioinks for bioprinting to create large-scale pre-vascularized 3D constructs.

5. Conclusions

In summary, we developed and characterized specialized Vascular Units (VUs) consisting of endothelial progenitor cells and organ-specific intestinal fibroblasts. We demonstrated their potential to serve as building blocks for the formation of lumenized capillaries and subsequent establishment of microvascular 3D networks within a hydrogel matrix. Using a customized biomimetic VoC, we showed the ability of VUs-derived capillaries to anastomose with larger-diameter vessels and become perfused. Lastly, by integrating VUs with IOs in the VoC, we illustrated how VUs can act as "vascularization bridges," extending a microvascular network throughout the hydrogel bulk and supporting the formation of a vascularized stroma around multiple organoids within a single microfluidic device. The versatility of VUs as tools in vascularization strategies opens a wide array of possibilities in tissue engineering, ranging from regenerative medicine to advanced microphysiological in vitro systems.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The work was developed under the scope of the EndoSWITCH project (PTDC/BTMORG/5154/2020), supported by the Portuguese Foundation for Science and Technology (FCT), and the REMODEL project (European Union's Horizon 2020 research and innovation programme, grant agreement 7857491). The authors thanks FCT for Iasmim Orge's PhD scholarship SFRH/BD/2020.07458 and Sílvia Bidarra's research contract DL 57/2016/CP1360/CT0006. The authors also acknowledge the support of i3S Scientific Platforms: "Bioimaging" member of the PPBI (Grant No: PPBI-POCI-01-0145-FEDER-022122), "Biointerfaces and Nanotechnology" (Grant No: UID/BIM/04293/2019) and "BioSciences Screening" (member of the PT-OPENSREEN (NORTE-01-0145-FEDER-085468) and PPBI (PPBI-POCI-01-0145-FEDER-022122)).

Data availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

1. Augustin, H. G.; Koh, G. Y., Organotypic vasculature: From descriptive heterogeneity to functional pathophysiology. *Science* **2017**, *357* (6353).
2. Palikuqi, B.; Nguyen, D. T.; Li, G.; Schreiner, R.; Pellegata, A. F.; Liu, Y.; Redmond, D.; Geng, F.; Lin, Y.; Gomez-Salinerio, J. M.; Yokoyama, M.; Zumbo, P.; Zhang, T.; Kunar, B.; Witherspoon, M.; Han, T.; Tedeschi, A. M.; Scottoni, F.; Lipkin, S. M.; Dow, L.; Elemento, O.; Xiang, J. Z.; Shido, K.; Spence, J. R.; Zhou, Q. J.; Schwartz, R. E.; De Coppi, P.; Rabbany, S. Y.; Rafii, S., Adaptable haemodynamic endothelial cells for organogenesis and tumorigenesis. *Nature* **2020**, *585* (7825), 426-432.
3. O'Connor, C.; Brady, E.; Zheng, Y.; Moore, E.; Stevens, K. R., Engineering the multiscale complexity of vascular networks. *Nat Rev Mater* **2022**, *7* (9), 702-716.
4. Rouwkema, J.; Khademhosseini, A., Vascularization and Angiogenesis in Tissue Engineering: Beyond Creating Static Networks. *Trends Biotechnol* **2016**, *34* (9), 733-745.
5. Wang, D.; Maharjan, S.; Kuang, X.; Wang, Z.; Mille, L. S.; Tao, M.; Yu, P.; Cao, X.; Lian, L.; Lv, L.; He, J. J.; Tang, G.; Yuk, H.; Ozaki, C. K.; Zhao, X.; Zhang, Y. S., Microfluidic bioprinting of tough hydrogel-based vascular conduits for functional blood vessels. *Sci Adv* **2022**, *8* (43), eabq6900.
6. Kong, Z.; Wang, X., Bioprinting Technologies and Bioinks for Vascular Model Establishment. *Int J Mol Sci* **2023**, *24* (1).
7. Dellaquila, A.; Le Bao, C.; Letourneur, D.; Simon-Yarza, T., In Vitro Strategies to Vascularize 3D Physiologically Relevant Models. *Adv Sci (Weinh)* **2021**, *8* (19), e2100798.
8. Bauman, E.; Feijao, T.; Carvalho, D. T. O.; Granja, P. L.; Barrias, C. C., Xeno-free pre-vascularized spheroids for therapeutic applications. *Sci Rep* **2018**, *8* (1), 230.
9. Feijao, T.; Neves, M. I.; Sousa, A.; Torres, A. L.; Bidarra, S. J.; Orge, I. D.; Carvalho, D. T. O.; Barrias, C. C., Engineering injectable vascularized tissues from the bottom-up: Dynamics of in-gel extra-spheroid dermal tissue assembly. *Biomaterials* **2021**, *279*, 121222.
10. Carvalho, D. T. O.; Feijão, T.; Neves, M. I.; da Silva, R. M. P.; Barrias, C. C., Directed self-assembly of spheroids into modular vascular beds for engineering large tissue constructs. *Biofabrication* **2021**, *13* (3).
11. Sinagoga, K. L.; Wells, J. M., Generating human intestinal tissues from pluripotent stem cells to study development and disease. *EMBO J* **2015**, *34* (9), 1149-63.
12. Yamaguchi, S.; Kanetaka, K.; Maruya, Y.; Higashi, M.; Kobayashi, S.; Hashiguchi, K.; Oohashi, F.; Sakai, Y.; Nakao, K.; Eguchi, S., Highly feasible procedure for laparoscopic transplantation of cell sheets under pneumoperitoneum in porcine model. *Surg Endosc* **2022**, *36* (6), 3911-3919.
13. Banno, K.; Yoder, M. C., Tissue regeneration using endothelial colony-forming cells: promising cells for vascular repair. *Pediatr Res* **2018**, *83* (1-2), 283-290.
14. Margolis, E. A.; Cleveland, D. S.; Kong, Y. P.; Beamish, J. A.; Wang, W. Y.; Baker, B. M.; Putnam, A. J., Stromal cell identity modulates vascular morphogenesis in a microvasculature-on-a-chip platform. *Lab Chip* **2021**, *21* (6), 1150-1163.
15. Strobel, H. A.; Moss, S. M.; Hoying, J. B., Methods for vascularization and perfusion of tissue organoids. *Mamm Genome* **2022**, *33* (3), 437-450.
16. Grebenyuk, S.; Ranga, A., Engineering Organoid Vascularization. *Front Bioeng Biotechnol* **2019**, *7*, 39.
17. Gomes, R. N.; Manuel, F.; Nascimento, D. S., The bright side of fibroblasts: molecular signature and regenerative cues in major organs. *NPJ Regen Med* **2021**, *6* (1), 43.
18. Ingram, D. A.; Mead, L. E.; Tanaka, H.; Meade, V.; Fenoglio, A.; Mortell, K.; Pollok, K.; Ferkowicz, M. J.; Gilley, D.; Yoder, M. C., Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. *Blood* **2004**, *104* (9), 2752-60.
19. Williams, P. A.; Stilhano, R. S.; To, V. P.; Tran, L.; Wong, K.; Silva, E. A., Hypoxia augments outgrowth endothelial cell (OEC) sprouting and directed migration in response to sphingosine-1-phosphate (S1P). *PLoS One* **2015**, *10* (4), e0123437.
20. Torres, A. L.; Bidarra, S. J.; Vasconcelos, D. P.; Barbosa, J. N.; Silva, E. A.; Nascimento, D. S.; Barrias, C. C., Microvascular engineering: Dynamic changes in microgel-entrapped vascular cells correlates with higher vasculogenic/angiogenic potential. *Biomaterials* **2020**, *228*, 119554.
21. Jochems, P. G. M.; van Berghenhenegouwen, J.; van Genderen, A. M.; Eis, S. T.; Wilod Versprille, L. J. F.; Wichers, H. J.; Jeurink, P. V.; Garssen, J.; Masereeuw, R., Development and validation of bioengineered intestinal tubules for translational research aimed at safety and efficacy testing of drugs and nutrients. *Toxicol In Vitro* **2019**, *60*, 1-11.

22. Chen, C.; Jochems, P. G. M.; Salz, L.; Schneeberger, K.; Penning, L. C.; van de Graaf, S. F. J.; Beuers, U.; Clevers, H.; Geijssen, N.; Masereeuw, R.; Spee, B., Bioengineered bile ducts recapitulate key cholangiocyte functions. *Biofabrication* **2018**, *10* (3), 034103.
23. Jansen, J.; De Napoli, I. E.; Fedecostante, M.; Schophuizen, C. M.; Chevtchik, N. V.; Wilmer, M. J.; van Asbeck, A. H.; Croes, H. J.; Pertijs, J. C.; Wetzels, J. F.; Hilbrands, L. B.; van den Heuvel, L. P.; Hoenderop, J. G.; Stamatialis, D.; Masereeuw, R., Human proximal tubule epithelial cells cultured on hollow fibers: living membranes that actively transport organic cations. *Sci Rep* **2015**, *5*, 16702.
24. Filova, E.; Brynda, E.; Riedel, T.; Chlupac, J.; Vandrovcova, M.; Svindrych, Z.; Lisa, V.; Houska, M.; Pirk, J.; Bacakova, L., Improved adhesion and differentiation of endothelial cells on surface-attached fibrin structures containing extracellular matrix proteins. *J Biomed Mater Res A* **2014**, *102* (3), 698-712.
25. Sato, T.; Vries, R. G.; Snippert, H. J.; van de Wetering, M.; Barker, N.; Stange, D. E.; van Es, J. H.; Abo, A.; Kujala, P.; Peters, P. J.; Clevers, H., Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* **2009**, *459* (7244), 262-265.
26. Gracz, A. D.; Puthoff, B. J.; Magness, S. T., Identification, isolation, and culture of intestinal epithelial stem cells from murine intestine. *Methods Mol Biol* **2012**, *879*, 89-107.
27. Ivanov, D. P.; Parker, T. L.; Walker, D. A.; Alexander, C.; Ashford, M. B.; Gellert, P. R.; Garnett, M. C., Multiplexing spheroid volume, resazurin and acid phosphatase viability assays for high-throughput screening of tumour spheroids and stem cell neurospheres. *PLoS One* **2014**, *9* (8), e103817.
28. Haase, R.; Royer, L. A.; Steinbach, P.; Schmidt, D.; Dibrov, A.; Schmidt, U.; Weigert, M.; Maghelli, N.; Tomancak, P.; Jug, F.; Myers, E. W., CLIJ: GPU-accelerated image processing for everyone. *Nat Methods* **2020**, *17* (1), 5-6.
29. Eglinger, J.; Karsjens, H.; Lammert, E., Quantitative assessment of angiogenesis and pericyte coverage in human cell-derived vascular sprouts. *Inflamm Regen* **2017**, *37*, 2.
30. Carpentier, G.; Berndt, S.; Ferratge, S.; Rasband, W.; Cuendet, M.; Uzan, G.; Albanese, P., Angiogenesis Analyzer for ImageJ - A comparative morphometric analysis of "Endothelial Tube Formation Assay" and "Fibrin Bead Assay". *Sci Rep* **2020**, *10* (1), 11568.
31. Trimm, E.; Red-Horse, K., Vascular endothelial cell development and diversity. *Nature Reviews Cardiology* **2023**, *20* (3), 197-210.
32. Chavkin, N. W.; Genet, G.; Poulet, M.; Jeffery, E. D.; Marziano, C.; Genet, N.; Vasavada, H.; Nelson, E. A.; Acharya, B. R.; Kour, A.; Aragon, J.; McDonnell, S. P.; Huba, M.; Sheynkman, G. M.; Walsh, K.; Hirschi, K. K., Endothelial cell cycle state determines propensity for arterial-venous fate. *Nature Communications* **2022**, *13* (1), 5891.
33. Fleischer, S.; Tavakol, D. N.; Vunjak-Novakovic, G., From arteries to capillaries: approaches to engineering human vasculature. *Adv Funct Mater* **2020**, *30* (37).
34. Traore, M. A.; George, S. C., Tissue Engineering the Vascular Tree. *Tissue Eng Part B Rev* **2017**, *23* (6), 505-514.
35. Muhl, L.; Genove, G.; Leptidis, S.; Liu, J.; He, L.; Mocci, G.; Sun, Y.; Gustafsson, S.; Buyandelger, B.; Chivukula, I. V.; Segerstolpe, A.; Raschperger, E.; Hansson, E. M.; Bjorkegren, J. L. M.; Peng, X. R.; Vanlandewijck, M.; Lendahl, U.; Betsholtz, C., Single-cell analysis uncovers fibroblast heterogeneity and criteria for fibroblast and mural cell identification and discrimination. *Nat Commun* **2020**, *11* (1), 3953.
36. Walji, N.; Kheiri, S.; Young, E. W. K., Angiogenic Sprouting Dynamics Mediated by Endothelial-Fibroblast Interactions in Microfluidic Systems. *Adv Biol (Weinh)* **2021**, *5* (11), e2101080.
37. Vila Cuenca, M.; Cochrane, A.; van den Hil, F. E.; de Vries, A. A. F.; Lesnik Oberstein, S. A. J.; Mummery, C. L.; Orlova, V. V., Engineered 3D vessel-on-chip using hiPSC-derived endothelial- and vascular smooth muscle cells. *Stem Cell Reports* **2021**, *16* (9), 2159-2168.
38. Orellano, I.; Thomas, A.; Herrera, A.; Brauer, E.; Wulsten, D.; Petersen, A.; Kloke, L.; Duda, G. N., Engineering Vascular Self-Assembly by Controlled 3D-Printed Cell Placement. *Advanced Functional Materials* **2022**, *32* (52), 2208325.
39. Arslan, U.; Brescia, M.; Meraviglia, V.; Nahon, D. M.; van Helden, R. W. J.; Stein, J. M.; van den Hil, F. E.; van Meer, B. J.; Vila Cuenca, M.; Mummery, C. L.; Orlova, V. V., Vascularized hiPSC-derived 3D cardiac microtissue on chip. *Stem Cell Reports* **2023**, *18* (7), 1394-1404.
40. Torres, A. L.; Bidarra, S. J.; Pinto, M. T.; Aguiar, P. C.; Silva, E. A.; Barrias, C. C., Guiding morphogenesis in cell-instructive microgels for therapeutic angiogenesis. *Biomaterials* **2018**, *154*, 34-47.

41. Salameh, S.; Tissot, N.; Cache, K.; Lima, J.; Suzuki, I.; Marinho, P. A.; Rielland, M.; Soeur, J.; Takeuchi, S.; Germain, S.; Breton, L., A perfusable vascularized full-thickness skin model for potential topical and systemic applications. *Biofabrication* **2021**, *13* (3).
42. Sano, T.; Nakajima, T.; Senda, K. A.; Nakano, S.; Yamato, M.; Ikeda, Y.; Zeng, H.; Kawabe, J. I.; Matsunaga, Y. T., Image-based crosstalk analysis of cell-cell interactions during sprouting angiogenesis using blood-vessel-on-a-chip. *Stem Cell Res Ther* **2022**, *13* (1), 532.
43. Whisler, J. A.; Chen, M. B.; Kamm, R. D., Control of perfusable microvascular network morphology using a multiculture microfluidic system. *Tissue Eng Part C Methods* **2014**, *20* (7), 543-52.
44. Huuskes, B. M.; DeBuque, R. J.; Kerr, P. G.; Samuel, C. S.; Ricardo, S. D., The Use of Live Cell Imaging and Automated Image Analysis to Assist With Determining Optimal Parameters for Angiogenic Assay in vitro. *Front Cell Dev Biol* **2019**, *7*, 45.
45. de Graaf, M. N. S.; Vivas, A.; Kasi, D. G.; van den Hil, F. E.; van den Berg, A.; van der Meer, A. D.; Mummery, C. L.; Orlova, V. V., Multiplexed fluidic circuit board for controlled perfusion of 3D blood vessels-on-a-chip. *Lab Chip* **2022**, *23* (1), 168-181.
46. Wu, Y.; Zhou, Y.; Paul, R.; Qin, X.; Islam, K.; Liu, Y., Adaptable Microfluidic Vessel-on-a-Chip Platform for Investigating Tumor Metastatic Transport in Bloodstream. *Analytical Chemistry* **2022**, *94* (35), 12159-12166.
47. Vo, Q.; Carlson, K. A.; Chiknas, P. M.; Brocker, C. N.; DaSilva, L.; Clark, E.; Park, S. K.; Ajiboye, A. S.; Wier, E. M.; Benam, K. H., On-Chip Reconstitution of Uniformly Shear-Sensing 3D Matrix-Embedded Multicellular Blood Microvessel. *Advanced Functional Materials* *n/a* (n/a), 2304630.
48. Murphy, A. R.; Allenby, M. C., In vitro microvascular engineering approaches and strategies for interstitial tissue integration. *Acta Biomaterialia* **2023**, *171*, 114-130.
49. Liu, S.; Lin, Z., Vascular Smooth Muscle Cells Mechanosensitive Regulators and Vascular Remodeling. *J Vasc Res* **2022**, *59* (2), 90-113.
50. Ferrari, D.; Sengupta, A.; Heo, L.; Pethö, L.; Michler, J.; Geiser, T.; de Jesus Perez, V. A.; Kuebler, W. M.; Zeinali, S.; Guenat, O. T., Effects of biomechanical and biochemical stimuli on angio- and vasculogenesis in a complex microvasculature-on-chip. *iScience* **2023**, *26* (3), 106198.
51. Bonanini, F.; Kurek, D.; Previdi, S.; Nicolas, A.; Hendriks, D.; de Ruiter, S.; Meyer, M.; Clapés Cabrer, M.; Dinkelberg, R.; García, S. B.; Kramer, B.; Olivier, T.; Hu, H.; López-Iglesias, C.; Schavemaker, F.; Walinga, E.; Dutta, D.; Queiroz, K.; Domansky, K.; Ronden, B.; Joore, J.; Lanz, H. L.; Peters, P. J.; Trietsch, S. J.; Clevers, H.; Vulto, P., In vitro grafting of hepatic spheroids and organoids on a microfluidic vascular bed. *Angiogenesis* **2022**, *25* (4), 455-470.
52. Nashimoto, Y.; Hayashi, T.; Kunita, I.; Nakamasu, A.; Torisawa, Y. S.; Nakayama, M.; Takigawa-Imamura, H.; Kotera, H.; Nishiyama, K.; Miura, T.; Yokokawa, R., Integrating perfusable vascular networks with a three-dimensional tissue in a microfluidic device. *Integr Biol (Camb)* **2017**, *9* (6), 506-518.
53. Leung, C. M.; de Haan, P.; Ronaldson-Bouchard, K.; Kim, G.-A.; Ko, J.; Rho, H. S.; Chen, Z.; Habibovic, P.; Jeon, N. L.; Takayama, S.; Shuler, M. L.; Vunjak-Novakovic, G.; Frey, O.; Verpoorte, E.; Toh, Y.-C., A guide to the organ-on-a-chip. *Nature Reviews Methods Primers* **2022**, *2* (1), 33.
54. Broguiere, N.; Isenmann, L.; Hirt, C.; Ringel, T.; Placzek, S.; Cavalli, E.; Ringnald, F.; Villiger, L.; Zullig, R.; Lehmann, R.; Rogler, G.; Heim, M. H.; Schuler, J.; Zenobi-Wong, M.; Schwank, G., Growth of Epithelial Organoids in a Defined Hydrogel. *Adv Mater* **2018**, *30* (43), e1801621.
55. Magno, V.; Meinhardt, A.; Werner, C., Polymer Hydrogels to Guide Organotypic and Organoid Cultures. *Advanced Functional Materials* **2020**, *30* (48), 2000097.

SUPPORTING INFORMATION

VASCULAR UNITS AS ADVANCED LIVING MATERIALS FOR BOTTOM-UP ENGINEERING PERFUSABLE 3D MICROVASCULAR NETWORKS

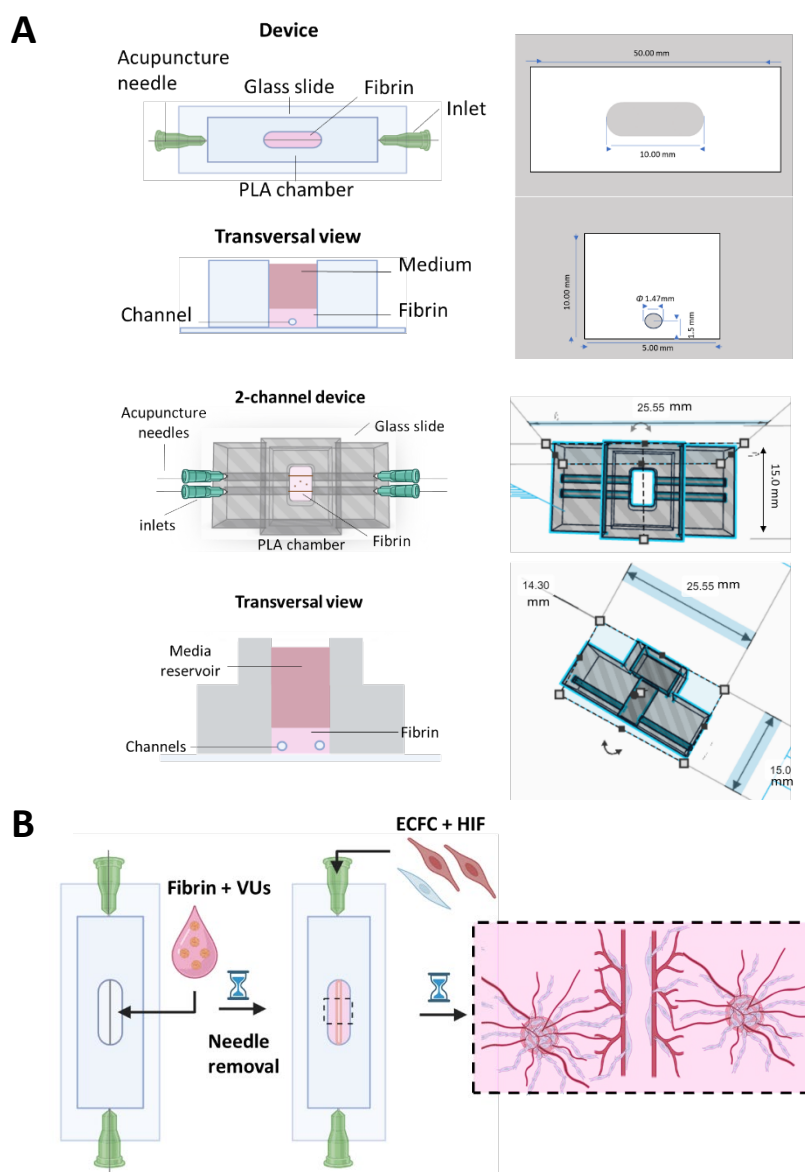


Figure S1. (A) Schematic design of the single channel and 2-channel microfluidic devices, showing both the top and transversal views and measurements of top and lateral sides. (B) Graphical abstract of the experimental approach where VUs were included in the device.

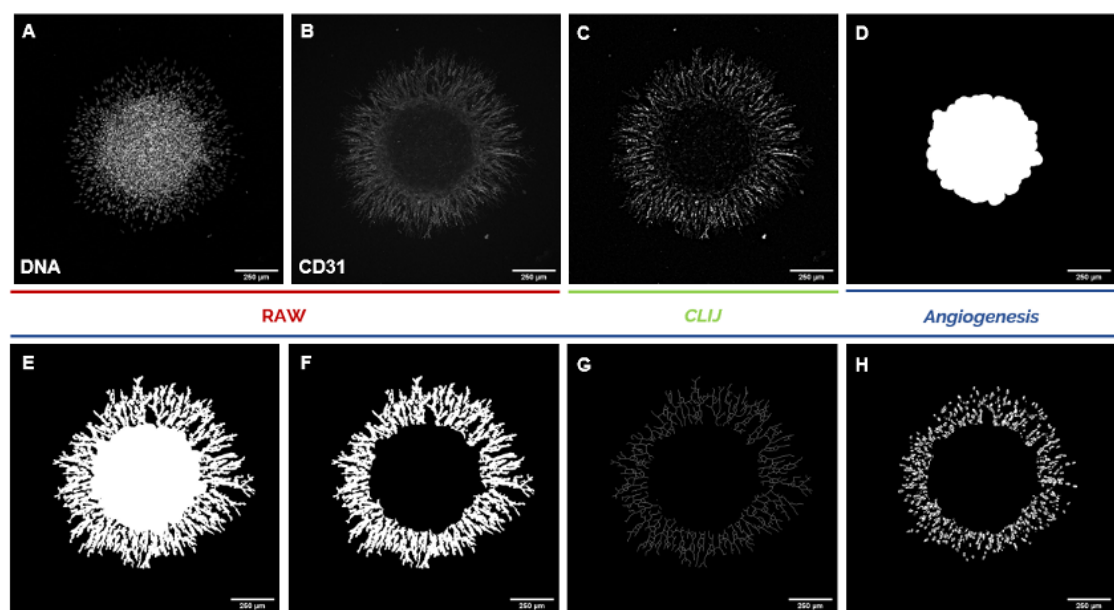


Figure S2. Morphological analysis of vessel sprouting from vascular units (VUs) embedded in fibrin. The sprouting analysis was conducted through semi-automated steps, including raw image processing (A-B) using CLIJ (C), followed by additional analysis with Angiogenesis plugins (D-H).

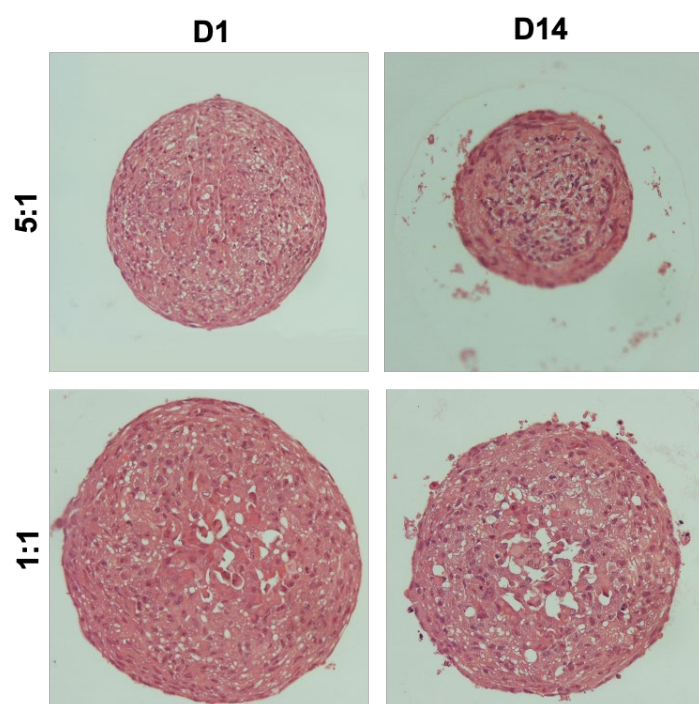


Figure S3. Brightfield images of histological sections stained with Hematoxylin and Eosin of 5:1 and 1:1 (hIF:ECFC) vascular units at days 1 and 14.

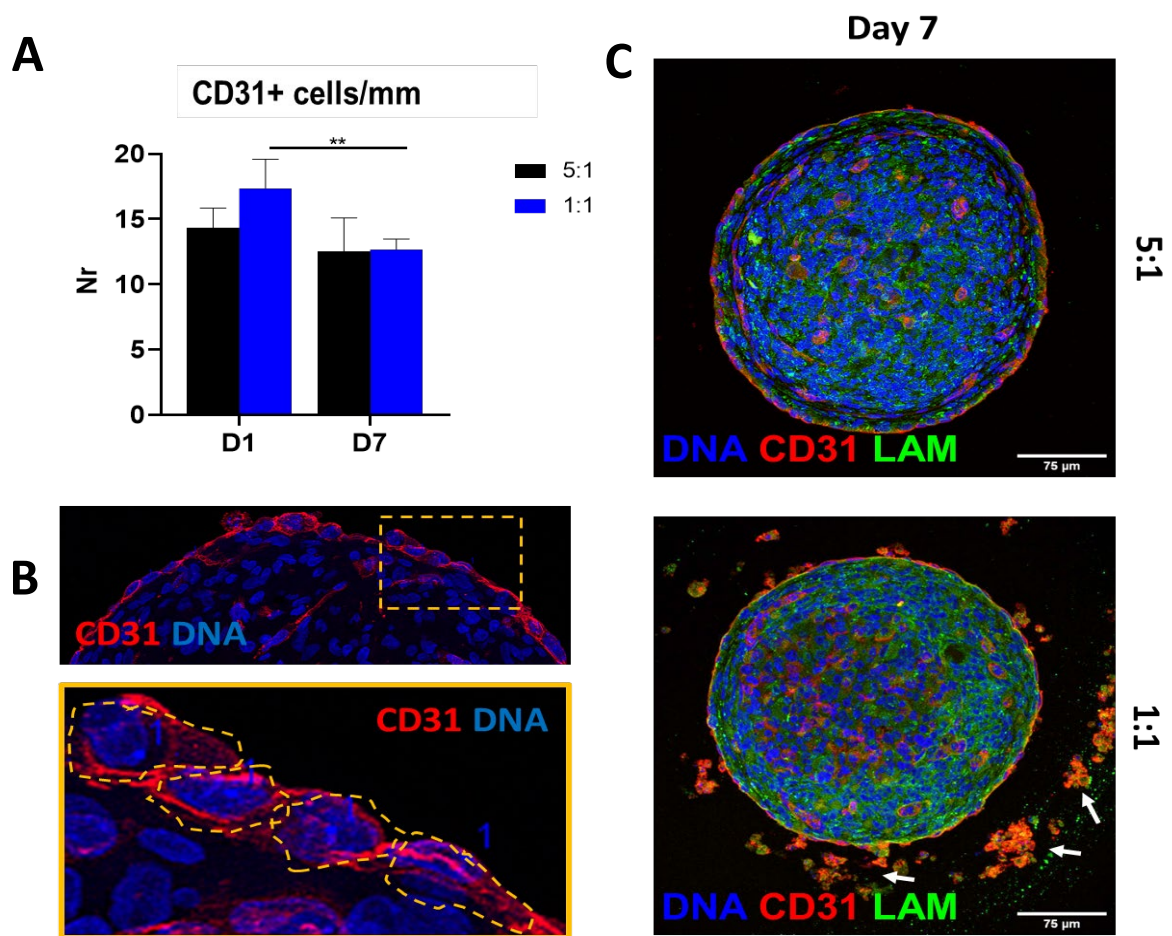


Figure S4. (A-B) Changes in the number of ECFC (CD31+ cells) in the outer layer of VUs at day 1 and 7. Number of CD31+ cells was quantified and normalized by VUs perimer. **(C)** Confocal microscopy images of 5:1 and 1:1 VUs, illustrating extensive ECM production in both ratios and CD31+ cell clusters being released from the peripheral corona after 7 days in 1:1 ratio (white arrows). Scale bars: 75 μ m.

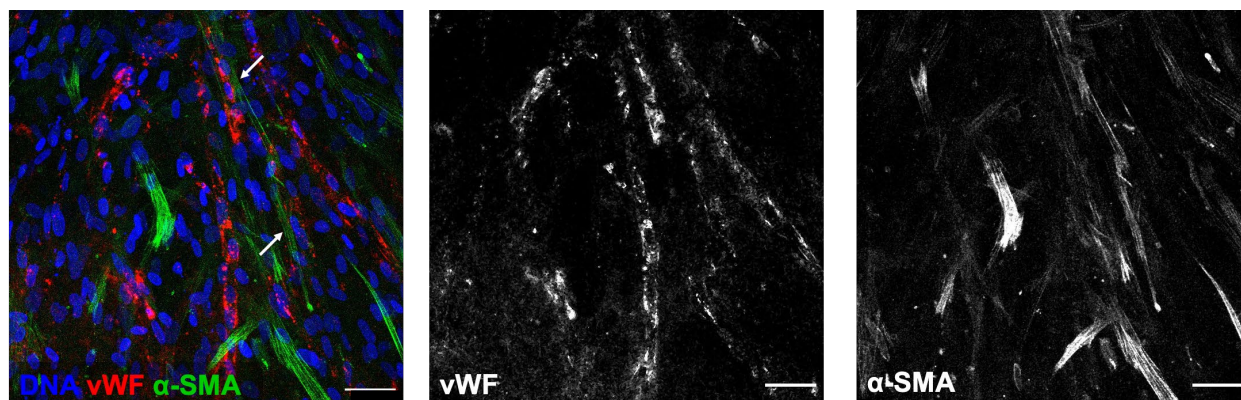


Figure S5. Immunofluorescence staining showing pericyte-like cells marked by α -SMA (white arrows) surrounding vWF+ capillary-like sprouts in VUs embedded in fibrin gel after 7 days. Scale bars: 50 μ m.

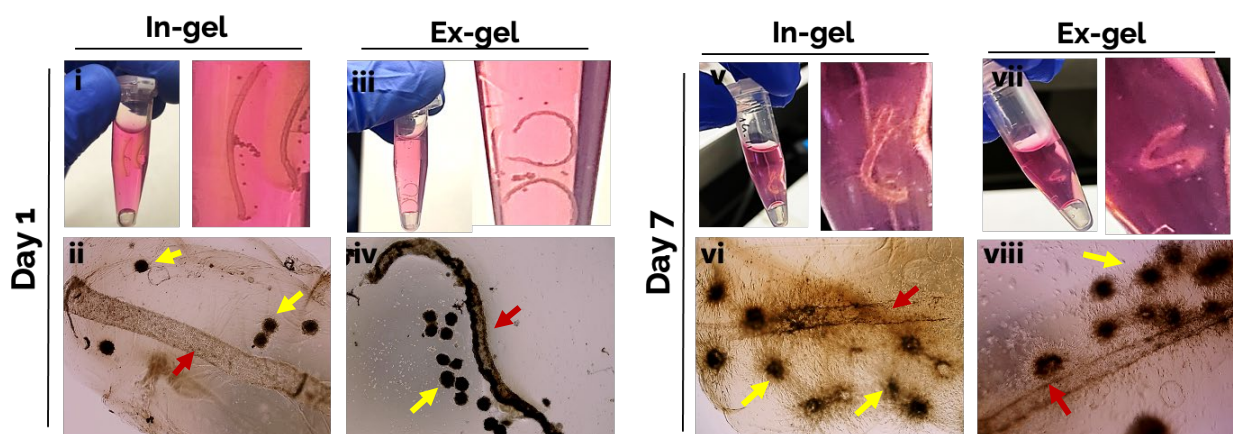


Figure S6. Macroscopic (i, iii, v, vii) and microscopic (ii, iv, vi, viii) images of the vascularized matrix removed from the chamber before (in-gel) and after (ex-gel) nattokinase treatment at days 1 and 7 post seeding. Red arrows: Vascular central channel. Yellow arrows: VUs.

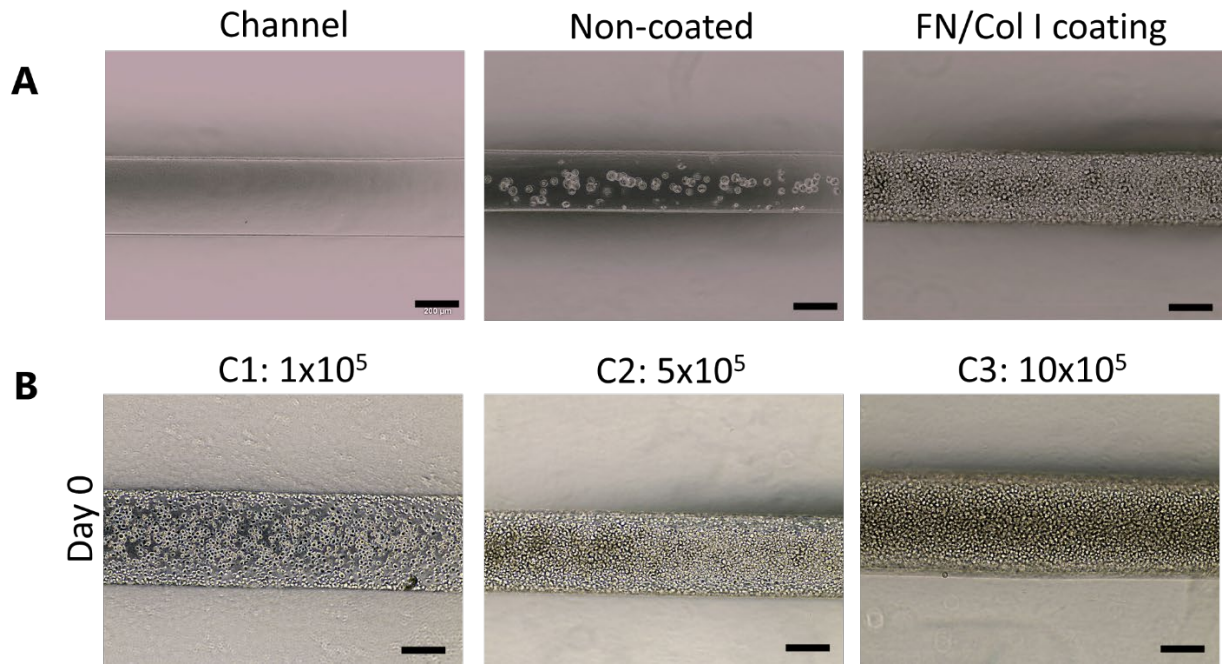


Figure S7. (A) Phase-contrast microscopy depicting the impact of non-coated and fibronectin/collagen I coated channels on cell adhesion. (B) Seeding (day 0) of coated channels with different cell seeding densities, C1: 1×10^5 , C2: 5×10^5 , C3: 10×10^5 . Scale bars: 200 μm .

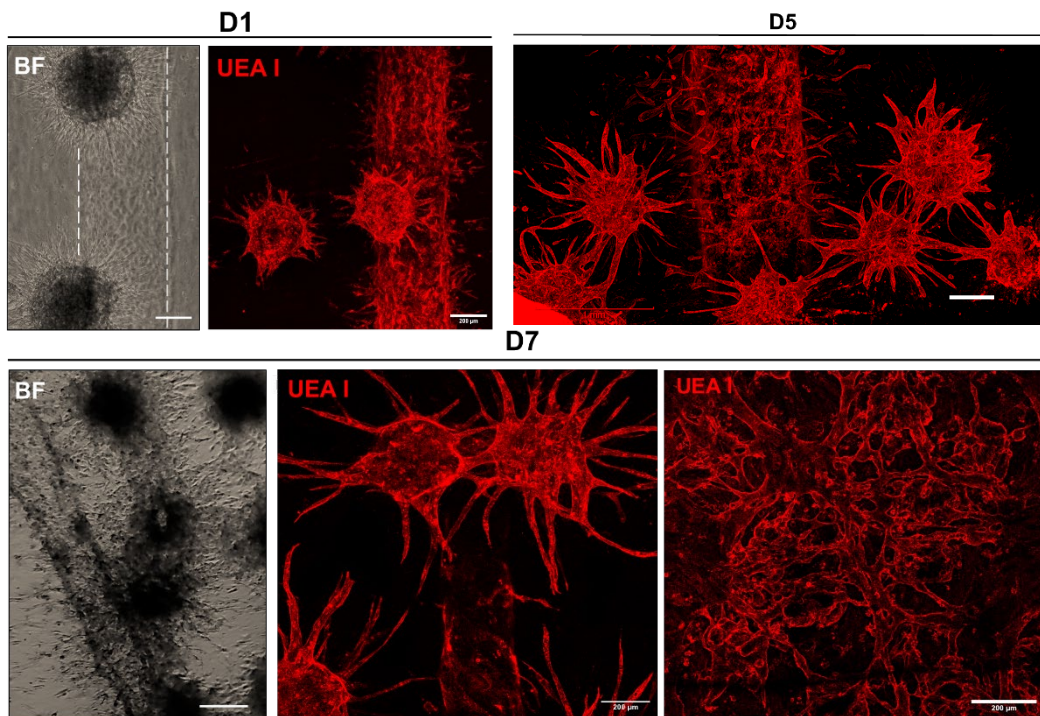


Figure S8. Introduction of VUs in VoC. phase contrast and fluorescent images of hydrogel-embedded VUs inside the perfused chamber at day 1, 5 and 7 (red: UEA I, white dashed lines: channel, scale bars: 200 μm).

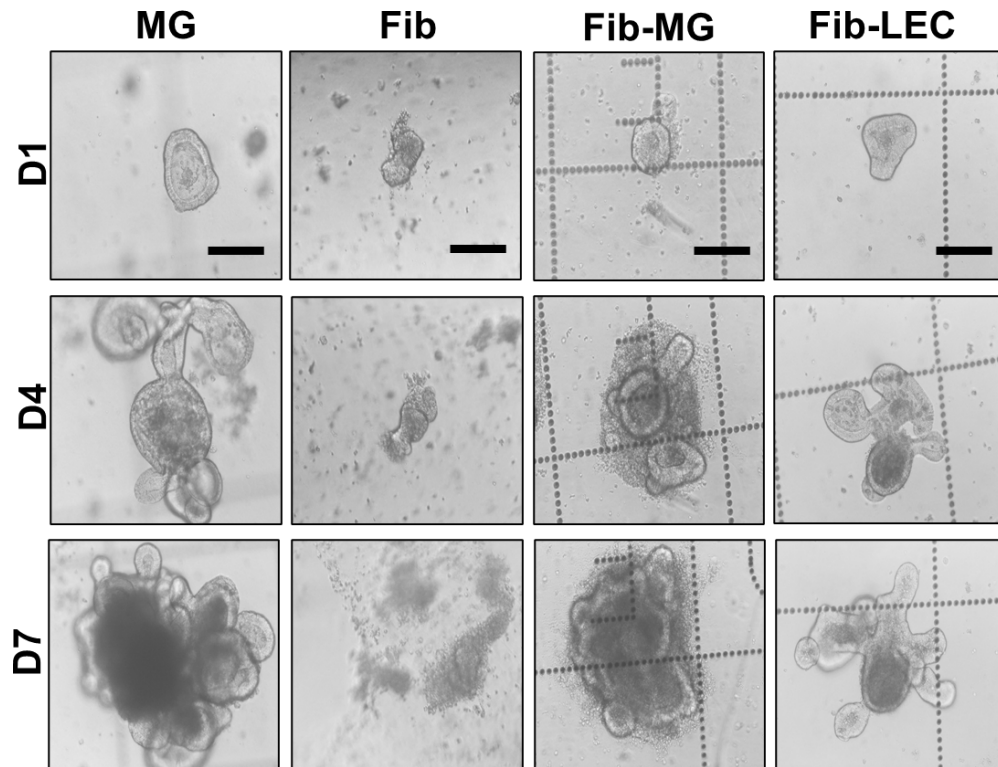


Figure S9. Supplemented fibrin hydrogels offer a viable alternative to MG for IOs culture. Representative phase-contrast images of morphological evolution of intestinal crypts embedded in different hydrogel matrices and formation of mature IOs after 7 days in culture. Scale bars: 100 μm .

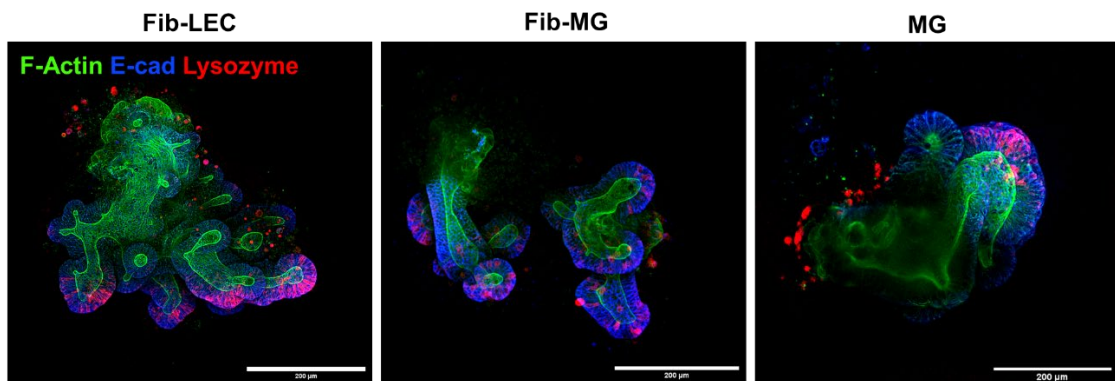


Figure S10. Organoids cultured in both supplemented hydrogels formulations expressed intestinal paneth cells marker lysozyme (F-actin:green, E-cad: Blue, Lysozyme: red). Scale bars: 200 μm .

Chapter IV

A peri-bile ductal fibrovascular stroma-on-chip for dissecting multicellular interactions in an inflammatory and fibrogenic liver microenvironment

Iasmim Orge^{1,2,3}, Álvaro Blanes⁴, Diana Nascimento¹, Ana Beatriz Sousa^{1,3}, Pedro Baptista⁴,
Cristina C. Barrias^{1,2,3}

¹ i3S – Instituto de Inovação e Investigação em Saúde, Rua Alfredo Allen, 208, 4200-135 Porto,
Portugal

² INEB - Instituto de Engenharia Biomédica, Universidade do Porto, Portugal

³ ICBAS-Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Portugal

⁴Aragón Health Research Institute (IIS Aragón), Zaragoza,
Spain

ABSTRACT

Cholangiopathies are a group of chronic liver diseases characterized by progressive biliary damage, inflammation, and fibrosis, often culminating in liver failure. A central feature of these conditions is the dynamic interplay between reactive cholangiocytes, stromal cells, and the peribiliary vascular plexus (PVP). However, in vitro models that recapitulate this complex microenvironment remain limited. Here, we present an integrated organ-on-chip platform designed to model the peri-bile ductal fibrovascular niche and investigate epithelial–stromal–vascular crosstalk under fibrogenic conditions. We first established a defined hydrogel system based on fibrin–laminin (FIB-LAM), which supported the expansion, differentiation, and functional maturation of intrahepatic cholangiocyte organoids (ICOs), offering a reproducible alternative to Matrigel. ICOs cultured in FIB-LAM showed enhanced epithelial organization, increased multi-lumen frequency, and expression of lineage-specific and functional markers. We then integrated ICOs into a microfluidic platform containing a FIB-LAM hydrogel with perfusable endothelial (ECFC) and hepatic stellate cells (HSC), enabling close spatial interaction between epithelial, vascular, and stromal compartments. This configuration mimicked early fibrogenic remodelling and revealed that reactive ICOs suppressed angiogenic sprouting, induced microvessel constriction, and triggered features of vascular destabilization. Proteomic profiling of conditioned media from co-culture chips (VS+ICO) revealed a unique secretome enriched for inflammatory, contractile, and anti-angiogenic signatures, including elevated expression of fibrogenic markers. Pathways enrichment analysis highlighted pathways linked to immune activation, cell contraction, cell death, and disease, aligning with the morphological changes observed on-chip. Altogether, this study establishes a physiologically relevant in vitro model of the peri-biliary niche that enables mechanistic investigation of cholangiocyte-enhanced fibrogenesis.

Keywords: Bile-duct, organ-on-chip, organoids, hepatic stellate cells, endothelial colony-forming cells.

1.Introduction

The hepatic biliary tree, lined by cholangiocytes, is central for liver function^[1]. Although cholangiocytes represent a minority of the liver's cellular population, they play critical roles in maintaining hepatic homeostasis. They are responsible for up to 30% of total bile flow in humans and play a vital role on the alkalization and fluidization of bile^[2, 3]. Moreover, cholangiocytes are important to liver regeneration (particularly when hepatocyte regeneration is impaired) and are known to be active on the regulation of both innate and adaptive hepatic immune responses. Significantly, their involvement in inflammation and fibrosis, particularly in cholestatic and other liver diseases, has emerged as a significant focus in hepatic pathobiology research^[1, 3].

Cholangiopathies comprise a spectrum of chronic intrahepatic and extrahepatic biliary tract disorders culminating in progressive cholestatic liver injury that may advance to fibrosis, cirrhosis, and liver failure, being often life-threatening and accounting for over 170,000 deaths worldwide in 2017^[4]. The rising global incidence and prevalence of cholestatic liver diseases underscore their significance as a pressing public health issue, but the development of effective therapies remains a critical unmet need ^[4]. In cholestatic liver diseases, hepatic stellate cells (HSCs) are among the first to undergo activation in response to bile acid accumulation and inflammatory signals. This is followed by the activation of periductular fibroblasts (PFs), which, together with HSCs, contribute to the fibrotic response leading to bile duct obstruction. Both cell types serve as key sources of fibrogenic myofibroblasts in the liver, driven by pro-inflammatory mediators and biliary injury. Cholestatic fibrogenesis is perpetuated by epithelial-mesenchymal crosstalk between cholangiocytes and myofibroblasts, which produce excessive extracellular matrix, driving structuring of the bile ducts and formation of fibrotic septa within the liver parenchyma. However, the exact relationship between the development of liver fibrosis and the progression of cholestatic liver disease remains elusive^[1].

The intrahepatic biliary tree is supported by a specialized portion of the liver's vasculature. Known as the peribiliary vascular plexus (PVP), this structure originates from the hepatic artery and drains into the portal vein and hepatic sinusoids. The PVP is strictly arranged around the intrahepatic bile ducts, playing a critical role in their pathophysiology. During development, the PVP lies adjacent to the nascent ductal plate, with structural changes in the PVP occurring later to accommodate the biliary tree's metabolic and functional demands. The close association of structural changes of the biliary epithelium with adaptive vascular responses has also been well documented in different disease settings ^[5]. For example, there is evidence linking the formation of new blood vessels around bile ducts as a response to inflammatory liver disease, which contributes to fibrosis and disease progression^[5]. On the other hand,

fibrosis also leads to excessive extracellular matrix (ECM) deposition, which increases tissue stiffness and density, generating mechanical tension that can impair or damage the microvasculature^[6].

Understanding the crosstalk between cholangiocytes, stromal/vascular cells, and their ECM requires advanced in vitro models that partially replicate the complexity of the cholestatic liver microenvironment. To advance cholangiocyte research, these models should aim to reproduce key features such as parenchymal–vascular–stromal interactions, cell–matrix dynamics, and inflammatory and pro-fibrotic signalling. While recent advances – including intrahepatic cholangiocyte organoids (ICOs), microfluidic systems ^[7-9], and co-culture approaches ^[10], have provided valuable insights, most remain limited in their ability to integrate all of these critical components in a single, physiologically relevant platform.

In the context of cholestatic fibrogenesis, a key objective is to reproduce periductular fibrosis and stricture formation to better mimic the pathological features of human disease. It has been proposed that a fibrotic-like microenvironment can drive cholangiocytes toward a pro-fibrogenic and pro-inflammatory phenotype, thereby exacerbating disease progression in a self-perpetuating cycle^[3]. This mechanism may, for instance, help explain the continued progression of biliary fibrosis in biliary atresia despite surgical relief of extrahepatic obstruction ^[3].

Here, we present the development of an innovative microfluidic device designed to recapitulate the peribiliary fibrovascular stroma and investigate its impact on cholangiocyte pathophysiology. The system features perfusable microvascular channels cast within a fibrin-based hydrogel that mimics a fibrovascular stroma, incorporating both endothelial cells and hepatic stellate cells. Intrahepatic cholangiocyte organoids are embedded within the same ECM-like hydrogel, allowing close spatial interactions among epithelial, stromal, and vascular components. This setup partially replicates a fibrotic-like microenvironment, enabling the study of cell–cell interactions relevant to cholestatic liver disease.

2. Materials and Methods

2.1. Intrahepatic Cholangiocyte Organoids (ICOs)

Isolation and expansion

Progenitor hepatic cells were obtained from healthy liver parenchyma biopsies. LGR5-Positive cells were isolated by a modified two-step collagenase perfusion technique, as described by Schneeberger et al^[11]. After isolation single cells were resuspended in Basement Membrane

Extract (BME) (Cultrex®) and plated in a 24-wells plate in drops of 50 µl. Human ICOs were grown in expansion medium (EM) composed of Advanced DMEM/F12 (Life Technologies) supplemented by 1% (v/v) P/S, 1% (v/v) 10 Mm HEPES (Gibco), and 1% (v/v) L-glutamine (Gibco), 50ug/ml (v/v) Primocin (InvivoGen), 1.25 mM N-acetylcysteine, 1x B27 without vitamin A (Gibco), 1x N2 (Gibco), 10% (v/v) R-Spondin-1–conditioned medium, 10 mM nicotinamide (Sigma-Aldrich), 100 ng/ml fibroblast growth factor 10 (FGF10) (Peprotech), 10 nM recombinant human (Leu15)-gastrin I (Merck Life Science), 10 µM forskolin (Sigma-Aldrich), 50 ng/ml epidermal growth factor (EGF) (Peprotech), 25 ng/ml hepatocyte growth factor (HGF) (Peprotech), 5 µM A8301 (transforming growth factor β inhibitor) (Stemcell Technologies) and 10 µM Y-27632 (Stemcell Technologies). Medium was changed every 2 days and ICOs were passaged every week. To passage or collect ICOs for other experiments, 1 mL of ice-cold basal Advanced DMEM/F12 was added to each well and domes were mechanically dissociated by pipetting up and down. The suspension was collected to a 15 mL tube and centrifuged for 5 min at 400 g, 4 °C. The pellet was then resuspended on TrypLE™ Express Enzyme (1X) (Gibco) and incubated at 37 °C for 5 minutes followed by a 5 min at 400 g centrifugation at 4 °C. Supernatant was carefully discarded, and the pellet was resuspended in the appropriate volume of BME. Passage ratio was usually 1-to-3 domes.

Hydrogel embedding

Laminin enriched fibrin hydrogels (FIB-LAM) were optimized according to previously published protocols ^[12-14]. Briefly, a fibrinogen solution (Sigma) was prepared in NaCl 0.9 % w/w mixed with aprotinin (Sigma) and diluted in EM to final concentrations of 2.5 mg/mL and 25 µg/mL, respectively. For ICOs embedding, these were added to the fibrinogen solution, mixed with thrombin (2 U/mL in PBS) at 4:5 v/v ratio and 2mg/mL 3-D Culture Matrix Laminin I (Cultrex®). Pre-gel solution was left 30 min at 37 °C for crosslinking.

Oscillation rheometry analysis

The rheological properties of the hydrogels were analysed by oscillatory shear rheometry (Kinexus Pro rheometer, Malvern) using a cone-on-plate (0.5°/40 mm) geometry. Each gel-precursor solution was prepared and immediately poured on the plate for analysis. A solvent trap filled with water was used to minimize sample drying. The evolution of the shear moduli, G' (storage, elastic component) and G'' (loss, viscous component), was recorded at 37°C as a function of time for 30 min. A frequency of 0.5 Hz and a strain of 1% were applied to maintain the linear viscoelastic regime. Three replicates were used for each condition.

Cholangiocyte differentiation

ICOs were removed from BME using ice-cold basal Advanced DMEM/F12, mechanically dissociated into cell clusters using a pipette tip, and transferred to either fresh BME mixed with 3 mg/ml rat-tail type I collagen (Merck Life Science) at a ratio of 2:1 or FIB-LAM hydrogels. Cultures were subsequently incubated at 37°C for 1 hour for polymerization followed by EM medium addition. After 3 days, the medium was changed to cholangiocyte differentiation medium (CDM) consisting of Advanced DMEM/F12 medium supplemented with 1% (v/v) P/S, 1% (v/v) HEPES, 1% (v/v) L-glutamine, 2% (v/v) B27 supplement without vitamin A, 1% (v/v) Insulin-Transferrin-Selenium (ITS) (Gibco), 1.25 mM NAC, 100 ng/ml FGF10, 10 nM gastrin, 50 ng/ml EGF, 25 ng/ml HGF, and 5 μ M A8301. Under differentiating conditions, cells were cultured for 6 days, and the medium was refreshed every other day.

Gene expression

For gene expression analyses, cell-loaded BME, BME-COL I or FIB-LAM hydrogels (on or out-chip) were dissociated by vigorously pipetting with ice-cold basal media. FIB-LAM hydrogels were additionally incubated with 3 mg/mL nattokinase solution for 20 min at 37 °C for fibrin degradation. RNA was extracted using the Quick-RNA MiniPrep (Zymo Research), following the manufacturer instructions. Subsequently, 500 ng of total RNA was reversed transcribed to single stranded cDNA using the Takara cDNA synthesis kit. Quantitative Real-Time PCR (qRT-PCR) was carried out using source RNA from 3 biological replicates (i.e., 3 individual experiments) for the target genes *AQP1*, *KRT7*, *LGR5*, *CFTR*, *GGT1*, *KRT19*, *HES1*, *EpCAM* and for endogenous control *GAPDH* using as probe sets Hs.PT.56a.27098577, Hs.PT.58.38355469, Hs.PT.58.227062, Hs.PT.58.3365414, Hs.PT.58.22838435, Hs.PT.58.4188708, Hs.PT.58.4181121, Hs.PT.58.39570895 and Hs.PT.51.1940505 (Applied Biosystems and Integrated DNA Technologies), respectively. Samples were run in triplicates in the ABI Prism 7000 Sequence Detection System under the following conditions: 95 °C for 20 s, followed by 40 cycles at 95 °C for 3 s and 60 °C for 30 s. The expression value for each target gene was normalized to GAPDH value for all the samples.

Rhodamine 123 transport assay

Cholangiocyte differentiated ICOs at day 6 in CDM were incubated with 100 μ M of Rhodamine 123 (Sigma-Aldrich) for 5 minutes at 37°C and then washed with basal media 3 times. Fresh CDM was added following the third wash and organoids were incubated at 37°C for another 40 minutes. To confirm that Rhodamine123 transfer accurately represented the activity of the membrane channel MultiDrug Resistance Protein 1 (MDR1), organoids were treated with 10 μ M Verapamil (Sigma-Aldrich) at 37°C for 30 minutes, followed by repeating the rhodamine

assay. Images were acquired using a Leica TCS SP5 AOBS spectral confocal microscope (Leica Microsystems, Germany).

Immunofluorescence

Hydrogel-embedded samples were analysed as whole-mount at pre-defined time points. Samples were washed with PBS and fixed with 4 % w/v PFA (30 min, RT). Then, gels were permeabilized with 0.5% v/v Triton X-100 for 30 min, blocked with 5% v/v BSA in PBS for 1 h at room temperature and incubated overnight with Alexa Fluor 647 Phalloidin (Invitrogen, 1:1000), Ulex Europaeus Agglutinin I Rhodamine (UEA I) (Vectors, 1:400) and the following primary antibodies diluted in 1 % w/v BSA: mouse anti-human CD31 (Dako, 1:100); rabbit anti-laminin (Sigma, 1:100); mouse anti-human alpha smooth muscle actin (α -SMA, Abcam, 1:200), mouse anti-human collagen IV (Dako, 1:100), rabbit anti-human von Willebrand factor (vWF) (Dako, 1:100), mouse anti-human β -catenin (β -CAT, BD Biosciences, 1:100); rabbit anti-E-cadherin (E-CAD, Cell signalling, 1:100), rabbit anti-Ki-67 (Abcam, 1:100), mouse anti-CK19 (Santa Cruz, 1:100) and rabbit anti-SOX9 (Sigma, 1:100). Samples were washed with 0.05 % v/v Tween-20 and incubated with the corresponding fluorochrome-conjugated secondary antibodies (Alexa 488 anti-mouse, Alexa 488 anti-rabbit, Alexa 594 anti-mouse and/or Alexa 594 anti-rabbit, Invitrogen, 1:500) and DAPI (1:500) for 4 h at RT.

Images were acquired using a Leica TCS SP5 AOBS spectral confocal microscope (Leica Microsystems, Germany) or in a high-content screening system equipped with a fully automated widefield/confocal fluorescence microscope (Opera Phenix Plus, Revvity).

2.2. Image analysis

Images were analyzed using ImageJ 1.50i, OrganoSeg^[15] and IMARIS softwares. OrganoSeg, an open-source software that integrates segmentation, filtering, and analysis for organoids was used to evaluate morphological characteristics of ICOs cultures on different hydrogels formulations. AngioTool Fiji plugin (0.6a, National Cancer Institute, USA), an open source, validated and reproducible software for quantifying vascular networks was used to calculate the Total Vessels Percentage area (at least three images per condition). For IMARIS imaging analysis, confocal images were saved as a single TIF file and converted to Imaris File Format Description (IMS) using the Imaris file converter. Converted files were read into Bitplane Imaris image visualization, segmentation and analysis software (Version 9.6.0–10.0, Oxford Instruments, Abington, UK). Surface rendering was performed for each channel of images. A surface detail ranging from 2 to 5 μ m was chosen followed by threshold and filter settings adjusted according to image intensities and quality. ImarisVantage statistical data visualization

module was used to calculate Shortest Distance of vessels staining surface to nearest neighbour HSCs surface to assess the distribution of HSCs on-chip.

2.3. Primary cell culture

Cells sources and maintenance

Human umbilical cord blood was obtained from healthy donors following a protocol approved by the Ethics Committee of CHUSJ (Centro Hospitalar Universitário de São João, Porto, Portugal). Endothelial colony forming cells (ECFC) were isolated from umbilical cord blood as previously described ^[16-18]. All blood donors were kept anonymous, so the need for written consent was waived. Cells were maintained in ECFC growth medium composed of MCDB-131 basal medium (BM) supplemented with 5 % v/v fetal bovine serum (FBS), 1 % v/v of penicillin-streptomycin (Pen/Strep), 2 mM L-glutamine, 1 µg/mL ascorbic acid, 0.2 µg/mL hydrocortisone, 2 ng/mL VEGF, 20 ng/mL IGF-1R, 10 ng/mL FGF, and 5 ng/mL EGF. Cells were used at passages 5 to 8. Primary human hepatic stellate cells (HSC) were kindly donated from Baptista's lab at Instituto de Investigación Sanitaria Aragón, Zaragoza, Spain. Cells were maintained on DMEM/F12 medium supplemented with 10% v/v FBS and 1% v/v Pen/Strep. Cells were used at passages 5 to 10. Both cell types were routinely cultured at 37 °C under a 5% v/v CO₂ humidified atmosphere and passage upon reaching ~80% confluence. The culture medium was changed every 2 days.

2.4. Microfluidic device

Fabrication of the microfluidic chamber and culture-on-chip

The polylactic acid (PLA) core of the chamber was 3D printed using the Fused Deposition Modelling method and the final design was optimized based on a previously reported set-up ^[9, 19, 20]. Briefly, melted PLA layers of 100 µm height were deposited in precise locations in a layer-by-layer approach to create the object. The chamber was then bonded to a glass coverslip with epoxy glue and left to dry overnight at room temperature. For the formation of the perfusable channels in the central reservoir of the microfluidic chamber, 3 acupuncture needles (300 µm diameter) were inserted inside the inlet and the central open-top reservoir was filled with the supplemented fibrinogen-thrombin solution for in situ gelification. For studies with ICOs, these were added to the hydrogel-precursor solution before loading into the chamber. After crosslinking, the needles were carefully removed, and the resulting paralleled hollow channels were coated with a fibronectin-collagen solution ^[21]. For endothelization, ECFC and HSC (10:1 ratio) suspended in EC growth medium were loaded into the channels at 2 x 10⁷/mL cell density in a volume of 10µL per channel. After 2 h, media was added to the

central well and lateral reservoirs. The chips were cultured under static conditions for the first 24 h after channel endothelialization and then 1mL and 500µL of media was added to each lateral reservoirs respectively to induce a gravity-driven flow. Devices were maintained at 37 °C under a 5% v/v CO₂ humidified atmosphere for 1-2 weeks. Media on the reservoirs was refreshed every 12 hours.

Perfusion assays

The basal fluorescence level of the system was acquired and then the fluorescent tracer FITC-Dextran 500 kDa (SIGMA) was added into one of the chip media lateral reservoirs (50 µL/chip) and the device was tilted to promote better gravity-driven flow to the outlet. After 5 min of perfusion, the device was imaged in a Leica TCS SP5 AOBS spectral confocal microscope (Leica Microsystems, Germany).

Flow cytometry and cell sorting

Hydrogels containing microvessels and organoids were scouped out from the chips and incubated with nattokinase 3mg/mL for 20 minutes at 37°C. Cells were washed with PBS with 2 % (v/v) FBS and incubated on TrypLE™ Express Enzyme (1X) (Gibco) and incubated at 37 °C for 5 minutes followed by a 5 min at 450 g centrifugation at 4 °C. For cell sorting, single-cell suspensions in FACS buffer (PBS with 2 % (v/v) FBS) were stained with the specific fluorochrome-conjugated antibodies CD31-FITC and CD90-APC for 30 min, at 4°C. Unstained cells were used as negative controls. Cell sorting was carried out using the FACS ARIA (BD Biosciences). CD31-FITC and CD90-APC negative cells (organoids) and positive (ECFC and HSC) were sorted and stored until further use.

Proteomics analysis

Conditioned media from chips was collected and total protein content was quantified with Pierce™ BCA Protein Assay Kit (ThermoFisher). Samples protein contents were uniformized to 100 µg in 300µL of ultrapure dH₂O. Protein identification and quantitation was performed by nanoLC-MS/MS. This equipment was composed by an Ultimate 3000 liquid chromatography system coupled to a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany). Raw data was processed using Proteome Discoverer 2.5.0.400 software (Thermo Scientific) and searched against the UniProt database for the Bos taurus and Homo sapiens proteomes and the NIST human spectral library.

To determine differentially expressed proteins, at least two unique peptides had to be identified for a protein to be included in the analysis. Contaminants from culture media and hydrogel

components were excluded. For the selection of upregulated proteins, the ratio was set to $FD \geq 1.25$; and for downregulated proteins it was set to $FD \leq 0.75$, with an adjusted p-value set to ≤ 0.05 . Heatmap was performed on heatmapper.ca software. Volcano plot analysis was performed with the GraphPad Prism 8.0 software version 8.01. Gene Ontology (GO) annotation and Reactome pathways analyses were performed on differentially expressed proteins on the Search Tool from EnrichR.

2.5. Statistical analysis

All the statistical analyses were performed using GraphPad Prism 8.3.0 software. For the data represented in the graphs depicted in Figure 1, 2-way ANOVA and Bonferroni's multiple comparisons test were used for the analysis. Shapiro-Wilk normality test was applied for the graphs of Figures 2-4, and the data was analyzed using one-way ANOVA Tukey's multiple comparisons test and the non-parametric Mann-Whitney test using a 99% confidence interval. Results comparing 2 groups were compared using t-test or Mann-Whitney in case of parametric or non-parametric distributions, respectively. Statistically significant differences are marked with * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), or **** ($p < 0.0001$).

3. Results and discussion

3.1 Culture in FIB-LAM hydrogel supports ICOs expansion

To create a more defined and pro-angiogenic 3D microenvironment for generating vascularized ICOs, a laminin-enriched fibrin-based matrix, referred to as FIB-LAM, was explored as an alternative to commonly used BME-based hydrogels. A similar FIB-LAM hydrogel has previously been shown to support the expansion of intestinal organoids and the formation of stable capillary-like structures^[12] As illustrated in Figure 1A, ICOs were isolated from liver biopsies and initially expanded in a BME matrix. Upon reaching confluency, ICOs were passaged and transferred to FIB-LAM hydrogels, where they were cultured in expansion medium (EM) for three days before the differentiation induction. ICOs cultured in BME-Col I served as controls based on previous published differentiation protocols^[8, 22]. Bright-field and confocal imaging revealed that ICOs cultured in EM developed a typical cystic morphology in both hydrogels, with comparable organoid formation efficiency (i.e., percentage of organoids formed from passaged fragments) (Figure 1B and Figure 1C). Moreover, as shown in Figure 1D i, ICO cultures in both matrices displayed polarized cellular organization, expressed β -catenin, and contained numerous Ki67⁺ proliferative cells. We further evaluated the expression of typical biliary organoid markers SOX9 and CK19. ICOs cultured in FIB-LAM showed positive staining for both specific markers (Figure 1D ii). The interaction between cholangiocytes and the extracellular matrix (ECM) is central for the development and maturation of the biliary tree

^[23]. Interestingly, we observed that the immediate contact of organoids with the FIB-LAM matrix, as early as day 1 post-passaging, triggered morphological changes characterized by the formation of internal segmentations, hereafter referred to as multi-lumen structures, which gradually disappeared over the course of culture in EM. While such structures were also detected in BME-Col I, they were present in a significantly lower percentage compared to FIB-LAM (Figure 1E i-ii). Although statistically different, the rheological analysis of the FIB-LAM and BME:Col I formulations showed that both hydrogels were considered as soft with G' around 15 Pa for FIB-LAM and around 25 Pa for BME:Col I (Figure 1F). Morphometric analysis using the OrganoSeg software revealed an increase in area, equivalent diameter, and solidity, alongside a decrease in skewness, in ICOs cultured in FIB-LAM from day 1 to day 3. These changes suggest a progression toward more cystic, single-lumen structures over time, with a reduction in internal complexity (Figure 1G). This multi-lumen morphology has been previously described in the literature as a characteristic feature of ICO differentiation, typically observed after 9 days of culture in differentiation medium^[24]. It has also been reported that lumen formation in ICOs is influenced by matrix properties ^[25]. For example, hydrogel stiffness and the concentration of cell- adhesive RGD (arginine-glycine-aspartate) motifs have been shown to influence cellular polarization and modulate the frequency of multi-lumen formation. Collectively, this suggests that contact with FIB-LAM may favour ICOs differentiation, although this matrix effect seems to be outweighed by culture in EM.

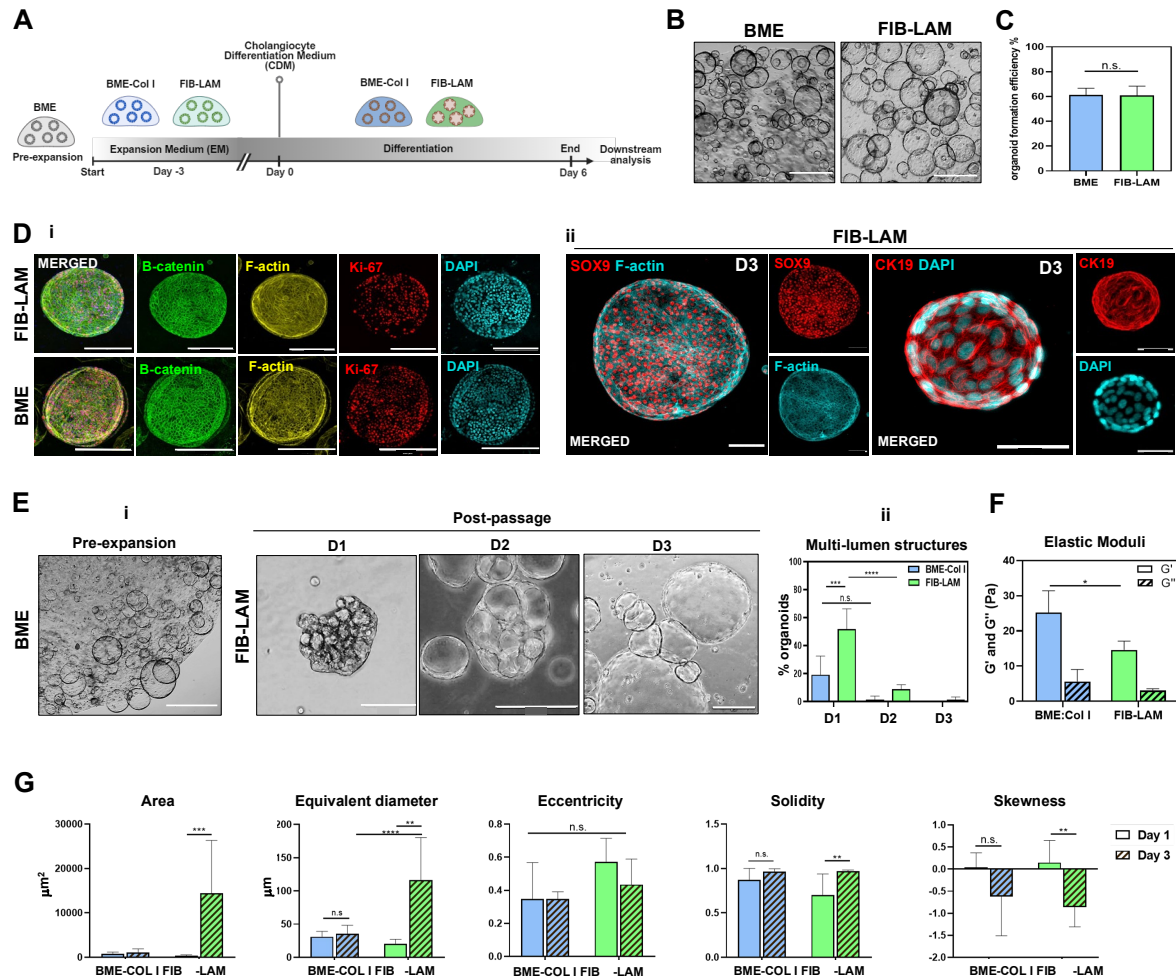


Figure 1. Expansion and characterization of ICOs cultured in BME-Col I or FIB-LAM matrices. (A) Schematic overview of the protocol for expansion and differentiation of ICOs in BME-Col I or FIB-LAM. Created with BioRender.com. (B) Representative bright-field images of confluent ICO cultures expanded in EM within BME or FIB-LAM matrices. Scale bars: 100 μm . (C) Quantification of organoid forming efficiency (%) 3 days after seeding in both matrices. (D) (i) Confocal images showing expression of epithelial (β -catenin: in green), proliferation (Ki-67: in red) and (ii) cholangiocyte markers (SOX9 and CK19: in red). Scale bars: 200 μm . (E) (i) Representative bright-field image of ICOs pre-expanded in BME matrix (Scale bar: 100 μm), and the morphological changes observed after 1- and 3-days post-passage after transferring to the FIB-LAM matrix (Scale bars: 50 μm). (ii) Quantification of the percentage of organoids with multiple lumens in FIB-LAM and BME-Col I matrices. (F) Rheological analysis of BME:Col I and FIB-LAM hydrogels showing G' and G'' . (G) Morphometric analysis of segmented bright-field images of ICOs in FIB-LAM and BME-Col I (1- and 3-days post-passage) using OrganoSeg software. In all graphs, statistically significant differences are marked with * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), or **** ($p < 0.0001$).

3.2. Culture in FIB-LAM hydrogel enhances functional ICOs differentiation

Following the characterization of ICO behavior on FIB-LAM hydrogels under expansion conditions, differentiation into bile-duct-like organoids was induced based on a previously established protocol [8, 22]. After two days in cholangiocyte differentiation medium (CDM), organoids cultured in both matrices exhibited comparable morphological changes, including increased density and thicker walls (Figure 2A), alongside a reduced growth rate (data not

shown). By day 6, however, these morphological alterations were more pronounced in organoids cultured within FIB-LAM hydrogels (Figure 2A). Confocal imaging and 3D rendering (Figure 2B) of β -catenin and F-actin highlighted distinct structural features in FIB-LAM cultures, where differentiating ICOs displayed segmented internal organization with multiple polarized lumens, reminiscent of structures observed in EM during early culture stages. Gene expression analysis (Figure 2C) highlighted differences in transcript levels between cultures maintained in EM and CDM, as well as between FIB-LAM and BME-Col I. The stemness marker *LGR5* exhibited the expected reduction following differentiation in both matrices. While ICOs in both matrices expressed the biliary markers *KRT7* and *KRT19*, their expression was notably lower in FIB-LAM-expanded organoids compared to the control under expansion conditions, further decreasing after differentiation. This aligns with previous reports demonstrating the formation of branching cholangiocyte organoids with mature profiles and functional tubular structures following bile duct differentiation *in vitro* [26]. Moreover, prior research has shown that *KRT19* expression is elevated in ICOs relative to primary cholangiocytes, suggesting that the observed reduction in *KRT19* may more accurately reflect the physiological expression pattern of cholangiocytes *in vivo* [27]. The increased expression of the functional marker *GGT1* in FIB-LAM hydrogels after differentiation, compared to the control, provides further evidence of enhanced organoid maturation. No significant differences were observed in the expression of *AQP1*, *HES1* and *CFTR*, although there was a tendency of decreased expression after differentiation in the *AQP1* and increased expression of *HES1* and *CFTR*. Previously it was demonstrated that ICOs can partially differentiate towards hepatocyte-like cells, reflecting their embryogenic origin and bipotent nature [28-30]. These studies collectively highlight the difficulty in achieving complete lineage commitment during differentiation protocols for ICOs, often resulting in a heterogeneous cell population. EpCAM is a marker for hepatic progenitor cells and remains expressed during biliary lineage commitment, particularly under Notch signalling activation, which drives differentiation into cholangiocytes [31]. Interestingly, ICOs differentiated in FIB-LAM hydrogels not only retained EpCAM expression but also showed a significant increase following the cholangiocyte differentiation protocol. Consistent with more differentiated states, the proportion of proliferative Ki67⁺ cells decreased when organoids were cultured in CDM compared to EM (Figure 2D). We next assessed the functional activity of differentiated ICOs. Cholangiocytes are known to mediate the reabsorption and secretion of bile acids, water, and small molecules in the liver, processes facilitated by various transmembrane channel proteins, including P-glycoprotein (also known as multidrug resistance protein 1, MDR1), which is localized on the apical surface of cholangiocytes [3]. To evaluate the transporter activity of differentiated ICOs, these were exposed to the fluorescent dye rhodamine 123, a substrate of MDR1, and its accumulation within the organoid lumens was monitored (Figure 2E). The specificity of the assay was confirmed by pre-treatment with

the calcium channel blocker verapamil, a MDRI inhibitor, that results in the accumulation of rhodamine 123 in the cytoplasm. In the absence of verapamil, the presence of rhodamine 123 was detected both in ICOs cultured in BME-Col I (single-lumen) and in FIB-LAM (multi-lumen), thereby confirming active transport through MDR1 and functionality. This suggests that both types of structures act as polarized epithelial barriers with fluid dynamic capabilities. Overall, FIB-LAM hydrogels provided a more defined and reproducible platform, supporting ICO expansion, promoting differentiation, and facilitating functional maturation, thereby offering a biologically relevant alternative to traditional BME-based matrices.

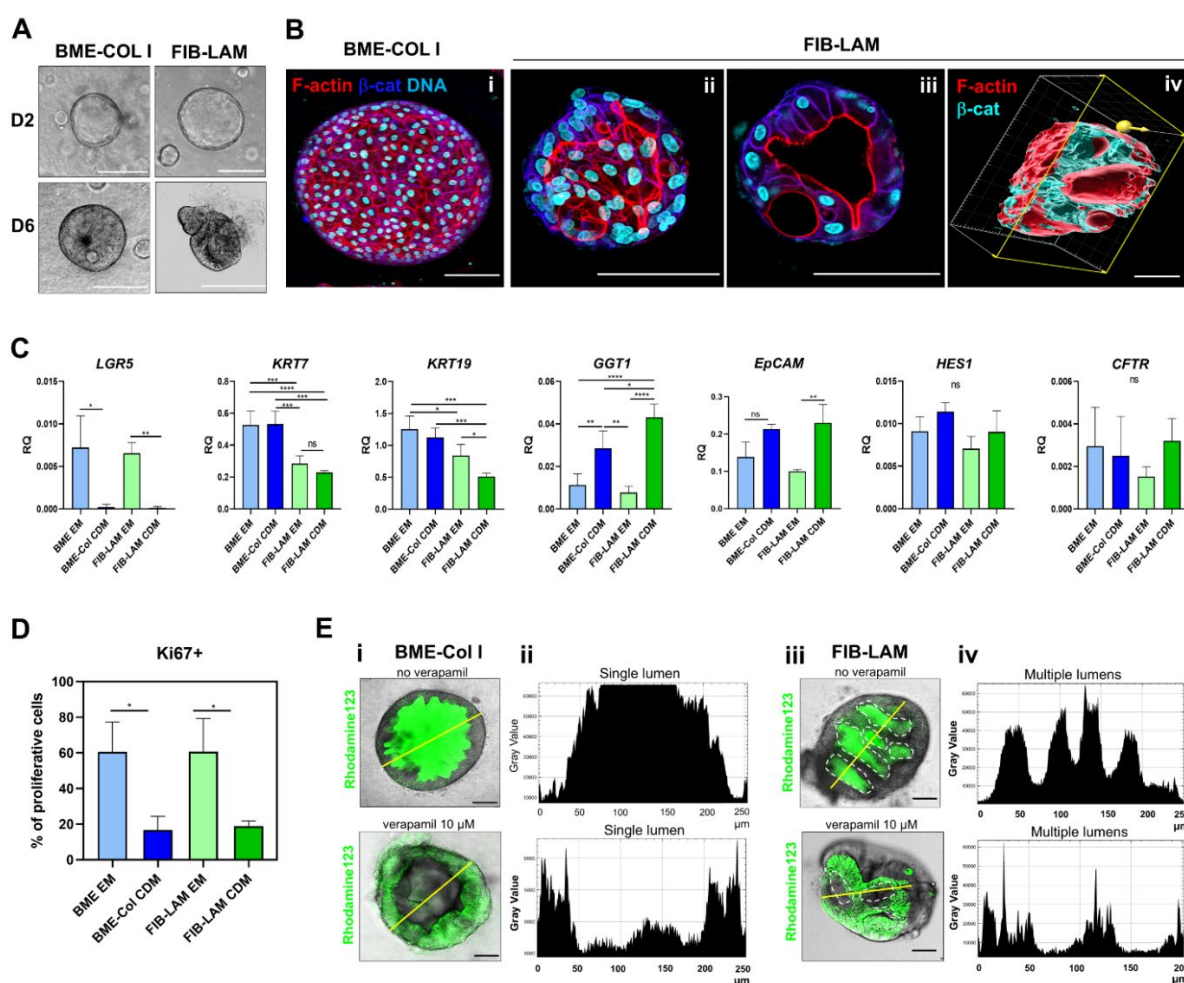


Figure 2. ICOs differentiation towards bile-duct-like organoids. (A) Representative bright-field images of organoids cultured in FIB-LAM and BME-Col I with CDM after 2 and 6 days. Scale bars: 100 μm. (B) Confocal images showing differentiated ICOs on BME-Col I (i) and on FIB-LAM (ii) matrices: maximum projection (iii) and middle stack view (iv) 3D segmented reconstruction showing a clipped central view of the organoid lumens. Scale bars: 100 μm (i-iii) and 40 μm (iv). (C) Gene expression analysis of cholangiocyte markers comparing ICOs cultured in EM or CDM (after 6 days) in both matrices. (D) Quantification of proliferative Ki67⁺ cells cultured in EM or CDM (after 6 days) in both matrices. (E) Brightfield and fluorescent images showing the P-glycoprotein transport function by active transport of rhodamine 123, with or without verapamil as an inhibitor of P-glycoprotein function (i and iii). Graphs depicting the fluorescence intensity along the yellow lines (ii and iv). Dashed lines: lumens, Scale bars: 50 μm. In all graphs, statistically significant differences are marked with * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), or **** ($p < 0.0001$).

3.3. Endothelial and hepatic stellate cells organize into a fibro-vascular stroma on-chip

To mimic the 3D microenvironment of the peribiliary fibro-vascular stroma, we designed a customized microfluidic device, building on our prior work ^[12]. The device consisted of three hollow microchannels cast within a FIB-based hydrogel and enclosed in an open-top PLA holder equipped with two medium reservoir compartments, enabling gravity-driven flow through the channels. Compared to pump-driven flow strategies, gravity-driven flow greatly simplifies device operation and enhances scalability, while promoting flow rates within a physiologically relevant range ^[32-34]. Microneedles were used to create the channels and subsequently removed following hydrogel crosslinking, yielding perfusable conduits with an average diameter of ~300 μm . To promote endothelialization, a cell suspension with endothelial colony-forming cells (ECFCs) and hepatic stellate cells (HSCs) at a 10:1 ratio was seeded into the microchannels at a density of 2×10^7 cells/mL. The experimental setup is illustrated in Figure 3A. Within the channels, ECFCs (CD31⁺ cells in red) formed a confluent monolayer lining the walls. From this layer, angiogenic sprouts emerged, invading the surrounding hydrogel and forming a hierarchical microvascular network that extended into the hydrogel bulk (Figure 3B). Notably, the HSCs (F-actin⁺/CD31⁻ cells in cyan) also migrated into the hydrogel matrix, where they contributed to the formation of stromal tissue surrounding the microvessels (Figure 3B-i). As depicted in Figure 3C, both the endothelialized microchannels and the microvascular network were enveloped by a basement membrane-like (BM) layer enriched with laminin (LAM, cyan) and collagen IV (COL IV, green), and they displayed well-defined lumens (arrows) (Figure 3C-ii and iv and Figure 3D-i). The formation of a BM layer around the microvessels is critical, as it supports endothelial organization, provides structural integrity to the developing microvascular network, and mimics the *in vivo* conditions of vascular maturation^[35, 36]. The functionality of the lumenized sprouts was assessed by infusing the microchannels with FITC-dextran (500 kDa). Evidence of flow throughout the microchannels and perfusate infiltration into the angiogenic sprouts was observed, with no apparent leakage (Figure 3D-ii). Stimulation with 10 ng/mL VEGF for 7 days resulted in an increased microvascular area, demonstrating that the system is responsive to pro-angiogenic mediators (Figure 3D-iii). The entire hydrogel construct – including the matrix, embedded microchannels, and microvascular network – could be easily retrieved from the central chamber, without compromising its overall integrity or collapsing the hollow microvessels (Figure 3E i) ^[12]. Once embedded in paraffin, the entire construct could be processed, sectioned, and stained with H&E, revealing an endothelial monolayer lining the channels (Figure 3E ii). While other studies described the development of microchip devices with open-top chambers ^[37-39], none has demonstrated the ability to preserve the microvascular network following sample harvesting, to the best of our knowledge, which is a distinctive feature of our system. This feature enhances

the versatility of the device, as the construct can be analysed off-chip, as a human tissue sample, for studies requiring structural integrity. Cells can also be easily isolated for downstream analyses, following enzymatically digestion of the hydrogel.

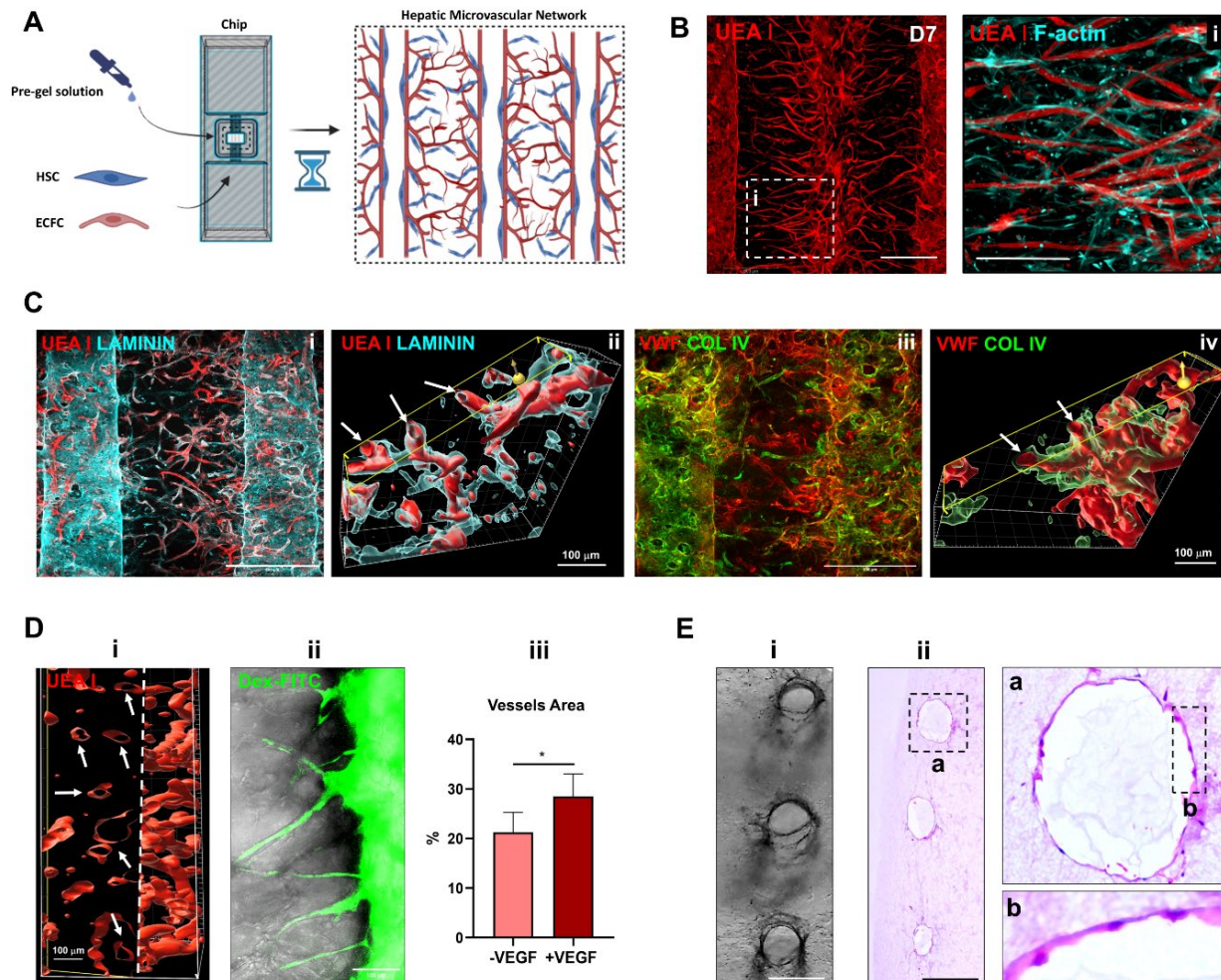


Figure 3. Fibro-vascular stroma on-chip. (A) Schematic overview of the microfluidic device. Created with BioRender.com. (B) Representative confocal image showing the formed vascular network from the chip channels after 7 days in culture. Scale bar: 500µm (i) Inset showing f-actin-stained HSCs (cyan blue) interaction with UEA I+ (red) vascular sprouts. Scale bar: 200 µm. (C) Laminin (i-ii) and collagen IV (iii-iv) deposition supporting the microvascular network showed by confocal images (i and iii) and 3D reconstructions with clipped view (ii and iv) Arrows: lumens, scale bars: 500 µm (i and ii) and 100 µm (ii and iv). (D) Orthogonal clipped view of UEA I+ microvessels showing lumenized sprouts. Arrows: Lumens. Scale bar: 100 µm (i). Brightfield and confocal image of Dextran FITC 500 kDa perfusion of a vascular channel and angiogenic sprouts. Scale bar: 100 µm (ii). Comparison between percentage of UEA I+ vascularized area on chips cultured with basal VEGF (2ng/mL final concentration) or supplemented VEGF (10ng/mL final concentration). * ($p < 0.05$) (iii). (E) Brightfield image showing a cross section of the bulk cell-laden hydrogel removed from the chip chamber. Scale bar: 500 µm (i). H&E staining of a sectioned the cell-laden hydrogel removed from the chip. Scale bar: 500 µm (ii).

3.4. The fibro-vascular stroma on-chip replicates a fibrogenic microenvironment

Canonically, hepatic stellate cell (HSC) activation is described as a two-phase process: initiation followed by perpetuation. In our model, we leveraged the well-established tendency of HSCs to spontaneously transition into an activated state when cultured in vitro, enabling us to mimic the initiation phase of fibrogenesis. Importantly, these cells are expected to remain responsive to microenvironmental signals and be further driven toward a more activated phenotype^{[40] [41, 42]}. The expression of fibrotic activation markers – α ACTA2 (α SMA), FAP, FN (fibronectin), Col1a1 (collagen type I), and MMP2, was analyzed to assess the activation state of HSCs relative to other mesenchymal cell types (Figure 4A). HSCs displayed higher expression of most markers compared to MSCs and, for selected genes, dermal fibroblasts FIBs, indicating a transition toward an activated phenotype. However, marker expression in HSCs remained consistently lower than in cancer-associated fibroblasts (CAFs, for most genes) and TGF β -stimulated HSCs (aHSCs), both of which exhibited strong upregulation across all genes. These results support that HSCs in our system are in an intermediate activation state, with fibrogenic potential that can be further exogenously modulated. On-chip, α SMA expression was confirmed by IF, highlighting widespread localization of activated HSCs (green) surrounding the perfusable vascular network stained with UEA1 (red). Despite being seeded at a lower density than ECFCs, HSCs proliferated extensively over five days in culture, forming a dense, interconnected stromal network that filled the area surrounding the central hydrogel compartment. These observations support the notion that upon integration into the chip, HSCs adopt a premature activation state, likely influenced by mechanical priming during prior 2D expansion. 3D image rendering and segmentation analysis revealed that HSCs were uniformly distributed throughout hydrogel, while also and maintained close spatial proximity to the microvessels (Figure 4C-i and ii). Notably, high-resolution imaging showed that a subset of HSCs wrapped around the vessel walls (Figure 4C iii), suggestive of a pericyte-like role in microvessel stabilization^[12-14, 43, 44].

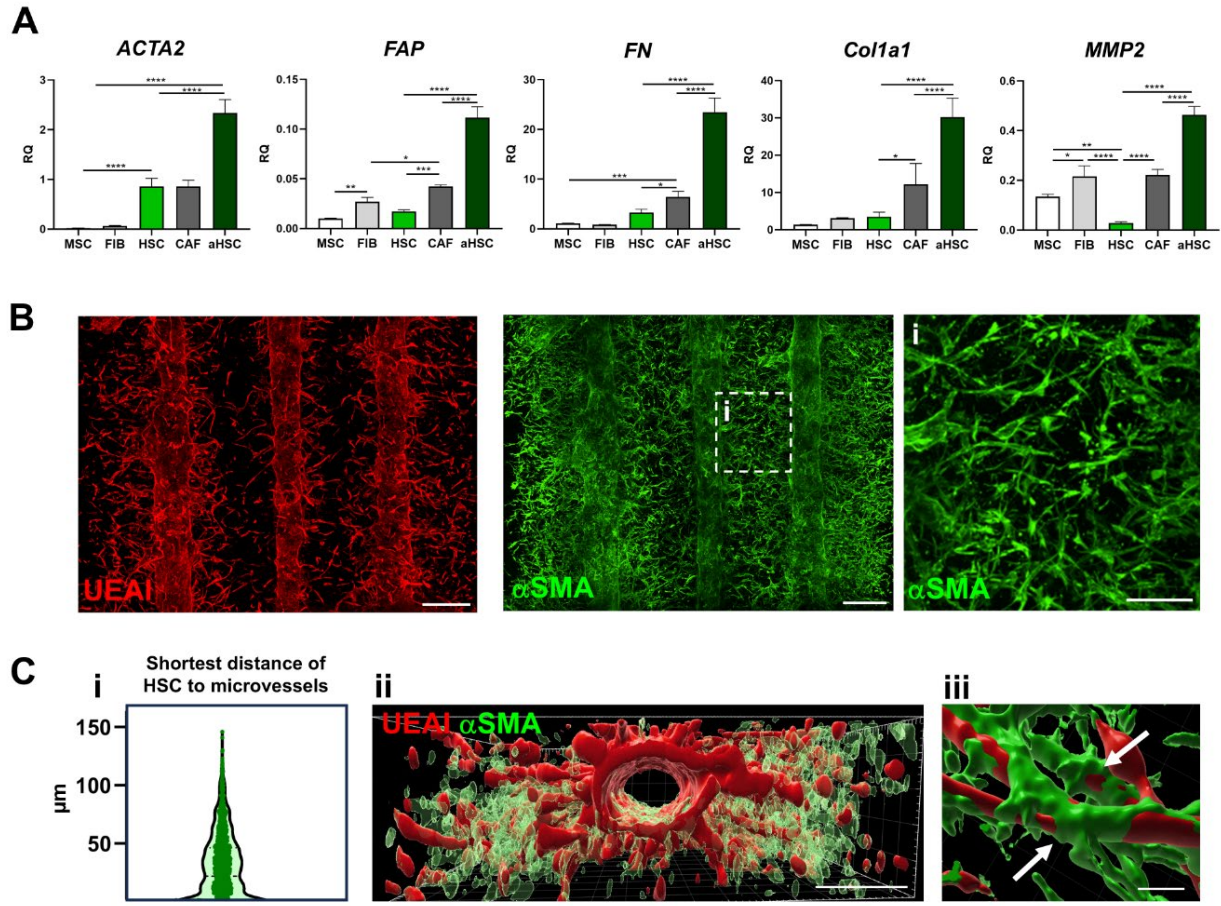


Figure 4. Activation profile of HSC on fibro-vascular stroma chip. (A) Gene expression analysis of activation markers on mesenchymal stromal cells (MSC), dermal fibroblasts (FIB), hepatic stellate cells (HSC), cancer-associated fibroblasts (CAF) and TGFβ-activated HSC (aHSC). (B) Immunostaining of fibro-vascular stroma on-chip with UEA I+ vessels in red and αSMA+ cells in green (i) inset highlighting αSMA+ cells. Scale bars: 500 μm; inset: 100 μm (C) Quantification of 3D segmented images measuring the distance between HSC and UEA I+ vessels (i). 3D reconstruction of channel cross-section showing UEA I+ ECFC and αSMA+ HSC organization. Scale bar: 500 μm (ii). 3D reconstruction showing HSC wrapping microvessels as perivascular supporting cells Scale bar: 50 μm (iii). In all graphs, statistically significant differences are marked with * (p < 0.05), ** (p < 0.01), *** (p < 0.001), or **** (p < 0.0001).

3.5. Crosstalk with reactive ICOs compromises microvessel integrity in a fibrovascular stroma

In a disease setting, the sustained interaction between a fibrogenic vascular stroma and reactive cholangiocytes may promote cholangiocyte activation and propagate an inflammatory feedback loop, thereby exacerbating both inflammation and fibrosis [45]. To investigate this crosstalk, we next examined the interaction between ICOs and a fibrovascular stroma using the microfluidic platform. ICOs were embedded in the pre-gel solution and randomly distributed around ECFC/HSC-perfused channels, as illustrated in the scheme of Figure 5A. By day 1, both the epithelial and stromal compartments developed as expected: ECFCs and HSCs adhered to the channel walls forming a continuous endothelium, and ICOs adopted their typical

cystic morphology. Notably, after 3 days, while the microchannels in control chips without ICOs presented some angiogenic sprouting and HSC outgrowth, such features were absent in chips containing ICOs (Figure 5B). Additionally, the microchannels appeared constricted, as if subjected to mechanical tension from the surrounding matrix and/or cellular components, with HSCs predominantly localizing near or around the channel walls (Figure 5B-i). By day 3, several ICOs were found in close proximity to the vessel walls (Figure 5B), with some extending cellular processes that appeared to attach directly to the endothelium (Figure 5C-i). These ICOs exhibited some morphological signs of differentiation, including denser cores and budding structures (Figure 5C-ii). Quantification of the distances between ICOs and surrounding microvessels revealed that the majority were located within 200 μm (Figure 5C-iii). Segmented 3D reconstructions of the vessels revealed signs of lumen collapse and vascular disruption (Figure 5C-iv and v). While the underlying mechanisms remain to be fully elucidated, the observed alterations in microvascular structures may, at least in part, result from paracrine signalling, mechanical stress, and/or matrix remodelling induced by nearby reactive cholangiocytes and activated HSCs.

Notably, cholangiocytes exhibit strong immunomodulatory properties and secrete a range of cytokines, including: interleukin-6 (IL-6), transforming growth factor-beta (TGF- β), and platelet-derived growth factor (PDGF); all of which are implicated in HSC activation, ductular reactions, and fibrogenesis^[3]. A recent study employed a microfluidic platform integrating an ICO-derived biliary channel and a HUVEC-lined vascular channel embedded in a collagen matrix with fibroblasts^[10]. In that system, cholangiocytes responded to IL-17 stimulation by secreting pro-inflammatory cytokines and facilitating the recruitment of immune cells through the vascular channel. However, vascular impairment, such as that observed in our system, was not observed. Additionally, another study investigating *in vitro* interactions between ICOs and HSCs reported that direct 3D co-culture led to HSC activation and enhanced ICO proliferation, accompanied by upregulation of Wnt signalling genes; hallmarks of the ductular reaction observed *in vivo*^[46]. These findings support the hypothesis that HSCs in our system may be responding to cholangiocyte-derived signals, and vice-versa, contributing to vascular abnormalities, potentially through the establishment of an inflammatory and contractile pro-fibrotic microenvironment.

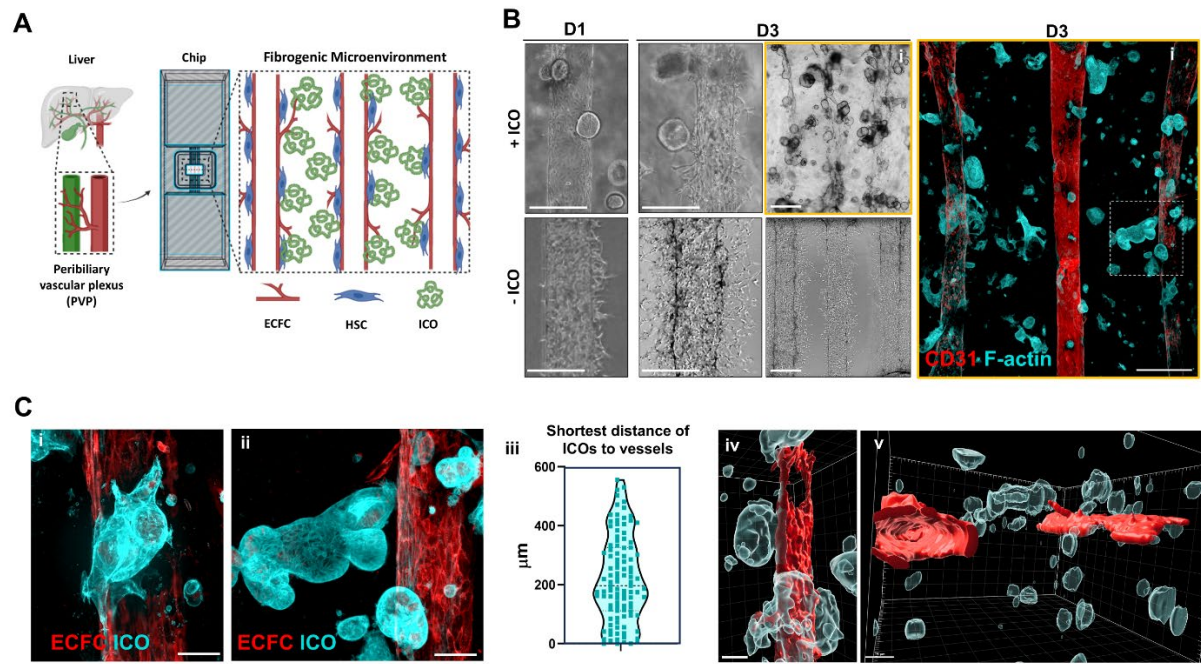


Figure 5. Multicellular model with ICOs, HSCs and ECFCs on-chip. (A) Graphical schematics of the PVP mimicking device configuration. (B) Brightfield and confocal imaging of chips with ICO and without ICOs at days 1 and 3 post seeding. Scale bars: 500 μ m. (C) (i) Amplification of confocal imaging showing an organoid (F-actin +, Cyan) attaching to the vessel wall (CD31+, red). (ii) Inset of figure 5B dashed white square showing ICOs budding protrusions. (iii) Quantification of the shortest distance from ICOs to CD31+ vessels structures on-chip. (iv-v) 3D segmented images showing ICOs distribution on-chip and close to damaged vessels. Scale bars: 150 μ m.

3.6. Peri-bile ductal fibrovascular stroma-on-chip uncovers secretory signals driving inflammation and fibrosis

To gain insight into the molecular mechanisms underlying the crosstalk between ICOs and the fibrovascular stroma, we performed proteomic analysis of the conditioned media collected after 3 days of culture. Secretome profiles were compared across three experimental group: the full chip set-up containing both the vascularized stroma and ICOs (VS+ICO), control chips containing only the fibrovascular stroma (VS), and chips containing only ICOs (ICO). This comparative approach aimed to identify paracrine signals and inflammatory or profibrotic mediators specifically enriched in the co-culture condition, thereby highlighting key pathways potentially driving stromal activation and fibrogenic progression. As shown in the principal component analysis (PCA) of the proteome profiles (Figure 6A), the three experimental conditions clustered distinctly, indicating clear differences in their secretome composition. The VS+ICO and VS-only groups clustered closely together, likely due to their similar vascular stromal (VS) cell content, which accounts for the majority of total cells in the chip. In contrast, the ICO-only group displayed a clearly distinct secretome profile. The partial overlap between VS+ICO and VS suggests that the vascular stroma exerts a dominant influence on the overall secretome. However, the distinct separation between these two groups also suggests that the

presence of ICOs may modulate the secretory activity of the stroma and/or reflect bidirectional crosstalk. Interestingly, we found some proteins that were exclusively present in each of the groups (Figure 6B). REG4 (Regenerating Islet-Derived Protein 4), ERP29 (Endoplasmic Reticulum Protein 29), and IGHV1-69V (Immunoglobulin Heavy Variable 1-69 Variant) were uniquely detected in the conditioned media from the ICO-only samples. REG4 is associated with epithelial regeneration, proliferation, and anti-apoptotic signalling, being frequently upregulated in response to tissue injury or repair^[47]. ERP29 is linked to endoplasmic reticulum (ER) stress and plays a role in the cellular response to protein-folding disturbances^[48]. The presence of these markers in ICOs on-chip, suggests that they may adopt a partially activated or stressed phenotype even prior to any interaction with the VS. This may reflect microenvironmental factors such as matrix confinement or medium composition, including the presence of pro-angiogenic growth factors, like VEGF. However, this must be verified through additional studies. COL4A2 and LTBP2 were exclusively detected in the VS samples, consistent with their roles in vascular remodelling and stabilization (Figure 6B). COL4A2, a key component of vascular basement membranes, supports vessel structure, while LTBP2 stabilizes elastic fibers and contributes to vessel wall integrity^[49]. Their presence in the VS condition may suggest a more mature and stable endothelial layer compared to the VS+ICO group, where vessel disruption was observed, as described in the previous section. Finally, MUC5AC, which encodes a secreted gel-forming mucin typically absent from normal intrahepatic bile ducts, was exclusively detected in the VS+ICO group (Figure 6B). Although not a direct effector of fibrosis, MUC5AC is frequently upregulated in fibrogenic and inflamed biliary environments^[50]. Its expression in the co-culture condition likely reflects a stress-induced response in ICOs triggered by interaction with the fibrogenic stroma, aligning with features of ductular reaction observed in chronic liver injury^[51, 52].

Interestingly, immunoglobulin proteins (IGHG1, IGHG2, IGKC and IGHM) were among the most abundant in ICO group (Figure 6C). The immunoregulatory and cytokine expression potential of cholangiocytes are described in the literature^[3], but evidence of aberrant immunoglobulin expression remains controversial and further investigation is necessary to confirm these observations. The most abundant proteins (Figure 6C) in the VS group (COL4A1, SERPINE1, PDXN and TIMP1) are related to angiogenesis modulation, cell migration, and basement membrane, confirming the angiogenic response observed in this condition (Figure 4), and also tissue remodelling, matrix deposition, and fibrosis, consistent with a fibrotic state^[53-55]. Finally, fibrogenic, inflammatory and anti-apoptotic proteins (VCAN, STC1, VIM, ARPC5, IQGAP1) are among the most abundant on VS+ICO group^[56-58] (Figure 6C). Similar to the PCA analysis, the global proteomic profile as visualized by the heatmap, revealed that the global expression patterns between the VS and VS+ICO groups were largely similar and different from the ICO-only condition (Figure 6D). Nonetheless, a total of 45

differentially expressed proteins were identified in the VS+ICO group compared to ICO group, where 27 were upregulated and 18 were downregulated. When compared to VS group, a total of 31 proteins were differentially expressed, where 16 were upregulated and 15 downregulated (Figure 6D).

The Volcano plots (Figure 6E) show the representation of the differentially expressed proteins in red (upregulated) and blue (downregulated) dots. Some of the most upregulated proteins in VS+ICO group when compared to ICO group are highlighted (CCT4, VIM, IQGAP1 and HMGA1) and were previously described to have increased expression during liver fibrosis and in the development of liver tumorigenesis ^[56, 59-61] (Figure 6E i). Downregulation of the REG4 gene has been linked to the development and progression of liver diseases, particularly non-alcoholic fatty liver disease (NAFLD) and hepatic steatosis^[62]. Intriguing, the immunoglobulins proteins IGHG1, IGHG2, IGKC and IGHM, were downregulated on VS+ICO but are among the most abundant proteins on ICO group (Figure 6C and 6E i). These findings suggest that ICOs adopt an immune-activated state^[3, 63] when exposed to on-chip culture conditions, even in the absence of fibrovascular stroma cells. This may reflect a shift toward an immune-suppressive or tolerogenic microenvironment, favouring fibrogenesis over immune surveillance or resolution^[64].

Although the damaging effects on the vascular compartment are evident in VS+ICO chips compared to the VS control (Figure 5), this is further supported by the upregulation of vascular inflammation/fibrosis-related proteins identified in the volcano plot, including AGR2, RPS3, and HSPD1^[65-67], as well as the downregulation of pro-angiogenic related proteins COL4A1 and LTPB2^[68] (Figure 6E ii).

Finally, enrichment analysis of upregulated pathways in the VS+ICO condition, compared to either ICO or VS controls, revealed strong associations with immune response and inflammation, tissue contraction, positive regulation of cell death, and disease (Figure 6F). These findings also align with the morphological alterations observed in the VS+ICO condition, as described in Figure 5.

Altogether, these molecular and morphological changes reflect the emergence of a pathological microenvironment characterized by inflammation, impaired angiogenesis, and matrix contraction, hallmarks of fibrogenic remodelling. This supports the utility of our platform in recapitulating key features of cholestatic liver disease and dissecting the detrimental crosstalk between reactive cholangiocytes, stromal, and vascular cells. However, to further support some of our hypotheses, complementary studies, such as transcriptomic analyses, are needed and are currently ongoing.

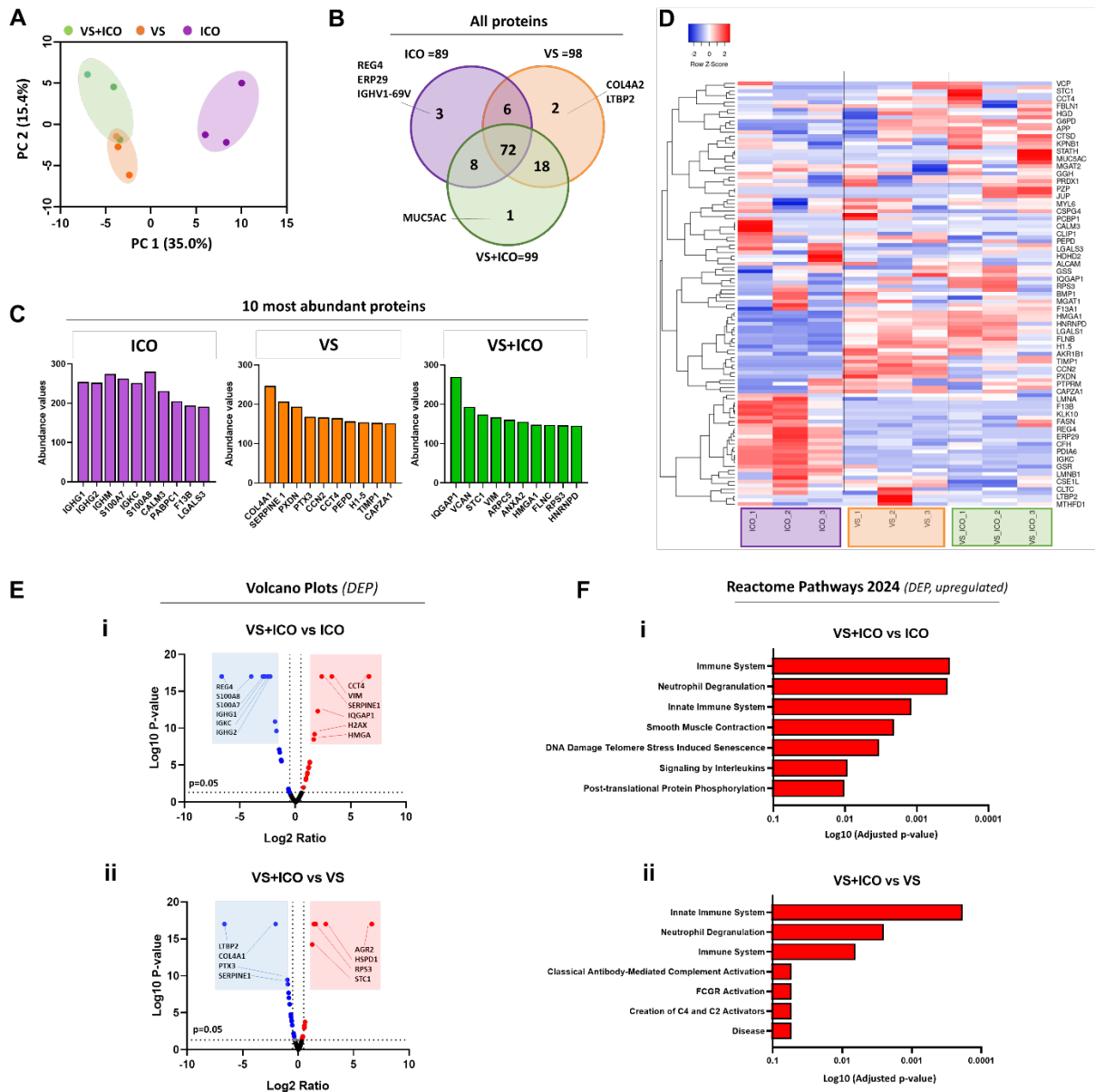


Figure 6. Proteomics analysis of chips secretome. (A) Principal component analysis of differentially expressed proteins. (B) Venn diagram comparing expressing of proteins from the different sample sources. (C) 10 most abundant proteins found on each group. (D) Heatmap with complete linkage clustering method with Pearson distance measurement method. (E) Volcano plots displaying the distribution of all proteins with relative protein abundance $\text{Log}_2(\text{FD}, \text{VS}+\text{ICO} \text{ versus } \text{ICO} \text{ or } \text{VS})$ plotted against the significance level ($-\text{Log}_{10}(\text{p-value})$, y-axis) and highlighting significantly ($\text{p-value} < 0.05$) up-regulated (red) and downregulates (blue) proteins. (F) Pathway enrichment analysis, showing related "Reactome Pathways", hierarchically organized by $-\log(\text{p-value})$ ($\text{p-value} < 0.05$) from EnrichR

4. Conclusions

We demonstrate that culturing intrahepatic cholangiocyte organoids (ICOs) in a fibrin–laminin (FIB-LAM) hydrogel supports both their expansion and functional differentiation, offering a defined and reproducible alternative to Matrigel. The FIB-LAM environment promotes epithelial organization and enhances lineage-specific marker expression, underscoring its suitability for

tissue-specific organoid culture. When integrated into a microfluidic platform containing a fibrovascular stroma, activated ICOs engage in bidirectional crosstalk that exacerbates inflammatory and fibrogenic responses. The presence of reactive ICOs, and their interactions with stromal and vascular cells, leads to upregulation of inflammatory and contractile pathways, vascular destabilization, and features consistent with fibrotic remodelling. While further studies and refinements are needed, these findings establish this model as a physiologically relevant in vitro system to investigate fibrogenic processes associated with cholangiopathies.

References

1. Jalan-Sakrikar, N.; Guicciardi, M. E.; O'Hara, S. P.; Azad, A.; LaRusso, N. F.; Gores, G. J.; Huebert, R. C., Central role for cholangiocyte pathobiology in cholestatic liver diseases. *Hepatology* **2024**.
2. Banales, J. M.; Prieto, J.; Medina, J. F., Cholangiocyte anion exchange and biliary bicarbonate excretion. *World J Gastroenterol* **2006**, *12* (22), 3496-511.
3. Banales, J. M.; Huebert, R. C.; Karlsen, T.; Strazzabosco, M.; LaRusso, N. F.; Gores, G. J., Cholangiocyte pathobiology. *Nat Rev Gastroenterol Hepatol* **2019**, *16* (5), 269-281.
4. Ouyang, G.; Liu, Q.; Wu, Y.; Liu, Z.; Lu, W.; Li, S.; Pan, G.; Chen, X., The global, regional, and national burden of gallbladder and biliary tract cancer and its attributable risk factors in 195 countries and territories, 1990 to 2017: A systematic analysis for the Global Burden of Disease Study 2017. *Cancer* **2021**, *127* (13), 2238-2250.
5. Thomson, J.; Hargrove, L.; Kennedy, L.; Demieville, J.; Francis, H., Cellular crosstalk during cholestatic liver injury. *Liver Res* **2017**, *1* (1), 26-33.
6. Sacchi, M.; Bansal, R.; Rouwkema, J., Bioengineered 3D Models to Recapitulate Tissue Fibrosis. *Trends Biotechnol* **2020**, *38* (6), 623-636.
7. Du, Y.; Khandekar, G.; Llewellyn, J.; Polacheck, W.; Chen, C. S.; Wells, R. G., A Bile Duct-on-a-Chip With Organ-Level Functions. *Hepatology* **2020**, *71* (4), 1350-1363.
8. Wang, Z.; Faria, J.; van der Laan, L. J. W.; Penning, L. C.; Masereeuw, R.; Spee, B., Human Cholangiocytes Form a Polarized and Functional Bile Duct on Hollow Fiber Membranes. *Front Bioeng Biotechnol* **2022**, *10*, 868857.
9. Chen, C.; Jochems, P. G. M.; Salz, L.; Schneeberger, K.; Penning, L. C.; van de Graaf, S. F. J.; Beuers, U.; Clevers, H.; Geijsen, N.; Masereeuw, R.; Spee, B., Bioengineered bile ducts recapitulate key cholangiocyte functions. *Biofabrication* **2018**, *10* (3), 034103.
10. Du, Y.; de Jong, I. E. M.; Gupta, K.; Waisbourd-Zinman, O.; Har-Zahav, A.; Soroka, C. J.; Boyer, J. L.; Llewellyn, J.; Liu, C.; Naji, A.; Polacheck, W. J.; Wells, R. G., Human vascularized bile duct-on-a chip: a multi-cellular micro-physiological system for studying cholestatic liver disease. *Biofabrication* **2023**, *16* (1).
11. Schneeberger, K.; Sánchez-Romero, N.; Ye, S.; van Steenbeek, F. G.; Oosterhoff, L. A.; Pla Palacin, I.; Chen, C.; van Wolferen, M. E.; van Tienderen, G.; Lieshout, R.; Colemonts-Vroninks, H.; Schene, I.; Hoekstra, R.; Verstegen, M. M. A.; van der Laan, L. J. W.; Penning, L. C.; Fuchs, S. A.; Clevers, H.; De Kock, J.; Baptista, P. M.; Spee, B., Large-Scale Production of LGR5-Positive Bipotential Human Liver Stem Cells. *Hepatology* **2020**, *72* (1), 257-270.
12. Orge, I. D.; Nogueira Pinto, H.; Silva, M. A.; Bidarra, S. J.; Ferreira, S. A.; Calejo, I.; Masereeuw, R.; Mihăilă, S. M.; Barrias, C. C., Vascular units as advanced living materials for bottom-up engineering of perfusable 3D microvascular networks. *Bioact Mater* **2024**, *38*, 499-511.
13. Feijao, T.; Neves, M. I.; Sousa, A.; Torres, A. L.; Bidarra, S. J.; Orge, I. D.; Carvalho, D. T. O.; Barrias, C. C., Engineering injectable vascularized tissues from the bottom-up: Dynamics of in-gel extra-spheroid dermal tissue assembly. *Biomaterials* **2021**, *279*, 121222.
14. Carvalho, D. T. O.; Feijão, T.; Neves, M. I.; da Silva, R. M. P.; Barrias, C. C., Directed self-assembly of spheroids into modular vascular beds for engineering large tissue constructs. *Biofabrication* **2021**, *13* (3).
15. Borten, M. A.; Bajikar, S. S.; Sasaki, N.; Clevers, H.; Janes, K. A., Automated brightfield morphometry of 3D organoid populations by OrganoSeg. *Sci Rep* **2018**, *8* (1), 5319.
16. Ingram, D. A.; Mead, L. E.; Tanaka, H.; Meade, V.; Fenoglio, A.; Mortell, K.; Pollok, K.; Ferkowicz, M. J.; Gilley, D.; Yoder, M. C., Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. *Blood* **2004**, *104* (9), 2752-60.
17. Williams, P. A.; Stilhano, R. S.; To, V. P.; Tran, L.; Wong, K.; Silva, E. A., Hypoxia augments outgrowth endothelial cell (OEC) sprouting and directed migration in response to sphingosine-1-phosphate (S1P). *PLoS One* **2015**, *10* (4), e0123437.
18. Torres, A. L.; Bidarra, S. J.; Vasconcelos, D. P.; Barbosa, J. N.; Silva, E. A.; Nascimento, D. S.; Barrias, C. C., Microvascular engineering: Dynamic changes in microgel-entrapped vascular cells correlates with higher vasculogenic/angiogenic potential. *Biomaterials* **2020**, *228*, 119554.
19. Jochems, P. G. M.; van Berghenhenegouwen, J.; van Genderen, A. M.; Eis, S. T.; Wilod Versprille, L. J. F.; Wichers, H. J.; Jeurink, P. V.; Garssen, J.; Masereeuw, R., Development and validation of bioengineered intestinal tubules for translational research aimed at safety and efficacy testing of drugs and nutrients. *Toxicol In Vitro* **2019**, *60*, 1-11.
20. Jansen, J.; De Napoli, I. E.; Fedecostante, M.; Schophuizen, C. M.; Chevtchik, N. V.; Wilmer, M. J.; van Asbeck, A. H.; Croes, H. J.; Pertijs, J. C.; Wetzels, J. F.; Hilbrands, L. B.; van den

- Heuvel, L. P.; Hoenderop, J. G.; Stamatialis, D.; Masereeuw, R., Human proximal tubule epithelial cells cultured on hollow fibers: living membranes that actively transport organic cations. *Sci Rep* **2015**, *5*, 16702.
21. Filova, E.; Brynda, E.; Riedel, T.; Chlupac, J.; Vandrovcova, M.; Svindrych, Z.; Lisa, V.; Houska, M.; Pirk, J.; Bacakova, L., Improved adhesion and differentiation of endothelial cells on surface-attached fibrin structures containing extracellular matrix proteins. *J Biomed Mater Res A* **2014**, *102* (3), 698-712.
 22. Wang, Z.; Ye, S.; van der Laan, L. J. W.; Schneeberger, K.; Masereeuw, R.; Spee, B., Chemically Defined Organoid Culture System for Cholangiocyte Differentiation. *Adv Healthc Mater* **2024**, *13* (30), e2401511.
 23. Fiorotto, R.; Mariotti, V.; Taleb, S. A.; Zehra, S. A.; Nguyen, M.; Amenduni, M.; Strazzabosco, M., Cell-matrix interactions control biliary organoid polarity, architecture, and differentiation. *Hepatol Commun* **2023**, *7* (4).
 24. Willemse, J.; van Tienderen, G.; van Hengel, E.; Schurink, I.; van der Ven, D.; Kan, Y.; de Ruiter, P.; Rosmark, O.; Westergren-Thorsson, G. G.; Schneeberger, K.; van der Eerden, B.; Roest, H.; Spee, B.; van der Laan, L.; de Jonge, J.; Verstegen, M., Hydrogels derived from decellularized liver tissue support the growth and differentiation of cholangiocyte organoids. *Biomaterials* **2022**, *284*, 121473.
 25. Funfak, A.; Bouzahir, L.; Gontran, E.; Minier, N.; Dupuis-Williams, P.; Gobaa, S., Biophysical Control of Bile Duct Epithelial Morphogenesis in Natural and Synthetic Scaffolds. *Front Bioeng Biotechnol* **2019**, *7*, 417.
 26. Roos, F. J. M.; van Tienderen, G. S.; Wu, H.; Bordeu, I.; Vinke, D.; Albarinos, L. M.; Monfils, K.; Niesten, S.; Smits, R.; Willemse, J.; Rosmark, O.; Westergren-Thorsson, G.; Kunz, D. J.; de Wit, M.; French, P. J.; Vallier, L.; JNM, I. J.; Bartfai, R.; Marks, H.; Simons, B. D.; van Royen, M. E.; Verstegen, M. M. A.; van der Laan, L. J. W., Human branching cholangiocyte organoids recapitulate functional bile duct formation. *Cell Stem Cell* **2022**, *29* (5), 776-794.e13.
 27. Aizarani, N.; Saviano, A.; Sagar, Mailly, L.; Durand, S.; Herman, J. S.; Pessaux, P.; Baumert, T. F.; Grün, D., A human liver cell atlas reveals heterogeneity and epithelial progenitors. *Nature* **2019**, *572* (7768), 199-204.
 28. Roos, F. J. M.; Wu, H.; Willemse, J.; Lieshout, R.; Albarinos, L. A. M.; Kan, Y. Y.; Poley, J. W.; Bruno, M. J.; de Jonge, J.; Bártfai, R.; Marks, H.; JNM, I. J.; Verstegen, M. M. A.; van der Laan, L. J. W., Cholangiocyte organoids from human bile retain a local phenotype and can repopulate bile ducts in vitro. *Clin Transl Med* **2021**, *11* (12), e566.
 29. Verstegen, M. M. A.; Roos, F. J. M.; Burka, K.; Gehart, H.; Jager, M.; de Wolf, M.; Bijvelds, M. J. C.; de Jonge, H. R.; Ardisasmita, A. I.; van Huizen, N. A.; Roest, H. P.; de Jonge, J.; Koch, M.; Pampaloni, F.; Fuchs, S. A.; Schene, I. F.; Luijck, T. M.; van der Doef, H. P. J.; Bodewes, F. A. J. A.; de Kleine, R. H. J.; Spee, B.; Kremers, G.-J.; Clevers, H.; Ijzermans, J. N. M.; Cuppen, E.; van der Laan, L. J. W., Human extrahepatic and intrahepatic cholangiocyte organoids show region-specific differentiation potential and model cystic fibrosis-related bile duct disease. *Scientific Reports* **2020**, *10* (1), 21900.
 30. Kaur, I.; Vasudevan, A.; Sanchez-Romero, N.; Sanyal, A.; Sharma, A.; Hemati, H.; Juneja, P.; Sharma, A.; Pla Palacin, I.; Rastogi, A.; Vijayaragavan, P.; Ghosh, S.; Ramakrishna, S.; Sarin, S. K.; Baptista, P. M.; Tripathi, D. M.; Kaur, S., In vivo transplantation of intrahepatic cholangiocyte organoids with decellularized liver-derived hydrogels supports hepatic cellular proliferation and differentiation in chronic liver injury. *J Mater Chem B* **2025**, *13* (3), 918-928.
 31. Safarikia, S.; Carpino, G.; Overi, D.; Cardinale, V.; Venere, R.; Franchitto, A.; Onori, P.; Alvaro, D.; Gaudio, E., Distinct EpCAM-Positive Stem Cell Niches Are Engaged in Chronic and Neoplastic Liver Diseases. *Front Med (Lausanne)* **2020**, *7*, 479.
 32. Bonanini, F.; Kurek, D.; Previdi, S.; Nicolas, A.; Hendriks, D.; de Ruiter, S.; Meyer, M.; Clapés Cabrer, M.; Dinkelberg, R.; García, S. B.; Kramer, B.; Olivier, T.; Hu, H.; López-Iglesias, C.; Schavemaker, F.; Walinga, E.; Dutta, D.; Queiroz, K.; Domansky, K.; Ronden, B.; Joore, J.; Lanz, H. L.; Peters, P. J.; Trietsch, S. J.; Clevers, H.; Vulto, P., In vitro grafting of hepatic spheroids and organoids on a microfluidic vascular bed. *Angiogenesis* **2022**, *25* (4), 455-470.
 33. Bonanini, F.; Dinkelberg, R.; Torregrosa, M. C.; Kortekaas, N.; Hagens, T. M. S.; Treillard, S.; Kurek, D.; van Duinen, V.; Vulto, P.; Bircsak, K., A microvascularized in vitro liver model for disease modeling and drug discovery. *Biofabrication* **2024**, *17* (1).
 34. Maurissen, T. L.; Spielmann, A. J.; Schellenberg, G.; Bickle, M.; Vieira, J. R.; Lai, S. Y.; Pavlou, G.; Fauser, S.; Westenskow, P. D.; Kamm, R. D.; Ragelle, H., Modeling early pathophysiological phenotypes of diabetic retinopathy in a human inner blood-retinal barrier-on-a-chip. *Nat Commun* **2024**, *15* (1), 1372.

35. Barreto-Ortiz, S. F.; Zhang, S.; Davenport, M.; Fradkin, J.; Ginn, B.; Mao, H. Q.; Gerecht, S., A novel in vitro model for microvasculature reveals regulation of circumferential ECM organization by curvature. *PLoS One* **2013**, *8* (11), e81061.
36. Snyder, Y.; Jana, S., Innovative Substrate Design with Basement Membrane Components for Enhanced Endothelial Cell Function and Endothelialization. *Adv Healthc Mater* **2024**, *13* (27), e2401150.
37. Jung, S.; Cheong, S.; Lee, Y.; Lee, J.; Lee, J.; Kwon, M.-S.; Oh, Y. S.; Kim, T.; Ha, S.; Kim, S. J.; Jo, D. H.; Ko, J.; Jeon, N. L., Integrating Vascular Phenotypic and Proteomic Analysis in an Open Microfluidic Platform. *ACS Nano* **2024**, *18* (36), 24909-24928.
38. Yrjänäinen, A.; Mesiä, E.; Lampela, E.; Kreutzer, J.; Vihinen, J.; Tornberg, K.; Vuorenpää, H.; Miettinen, S.; Kallio, P.; Mäki, A.-J., Barrier-free, open-top microfluidic chip for generating two distinct, interconnected 3D microvascular networks. *Scientific Reports* **2024**, *14* (1), 22916.
39. Kim, S.; Park, J.; Ho, J. N.; Kim, D.; Lee, S.; Jeon, J. S., 3D vascularized microphysiological system for investigation of tumor-endothelial crosstalk in anti-cancer drug resistance. *Biofabrication* **2023**, *15* (4).
40. Luo, N.; Li, J.; Wei, Y.; Lu, J.; Dong, R., Hepatic Stellate Cell: A Double-Edged Sword in the Liver. *Physiol Res* **2021**, *70* (6), 821-829.
41. Gandhi, C. R., Hepatic stellate cell activation and pro-fibrogenic signals. *Journal of Hepatology* **2017**, *67* (5), 1104-1105.
42. Haaker, M. W.; Goossens, V.; Hoogland, N. A. N.; van Doorne, H.; Wang, Z.; Jansen, J. W. A.; Kaloyanova, D. V.; van de Lest, C. H. A.; Houweling, M.; Vaandrager, A. B.; Helms, J. B., Early activation of hepatic stellate cells induces rapid initiation of retinyl ester breakdown while maintaining lecithin:retinol acyltransferase (LRAT) activity. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **2024**, *1869* (7), 159540.
43. Neves, S. C.; Sousa, A.; Nascimento, D. S.; Orge, I. D.; Ferreira, S. A.; Mota, C.; Moroni, L.; Barrias, C. C.; Granja, P. L., A hybrid construct with tailored 3D structure for directing pre-vascularization in engineered tissues. *Mater Today Bio* **2024**, *29*, 101291.
44. Asahina, K., Hepatic stellate cell progenitor cells. *J Gastroenterol Hepatol* **2012**, *27 Suppl 2* (Suppl 2), 80-4.
45. Tsuchida, T.; Friedman, S. L., Mechanisms of hepatic stellate cell activation. *Nature Reviews Gastroenterology & Hepatology* **2017**, *14* (7), 397-411.
46. Haaker, M. W.; Chang, J.-C.; Chung, B. K.; Pieper, T. S.; Noé, F.; Wang, T.; Geijsen, N.; Houweling, M.; Wolfrum, C.; Vaandrager, A. B.; Melum, E.; Spee, B.; Helms, J. B., Cellular Crosstalk Promotes Hepatic Progenitor Cell Proliferation and Stellate Cell Activation in 3D Co-culture. *Cellular and Molecular Gastroenterology and Hepatology* **2025**, *19* (5), 101472.
47. Zhang, J.; Zhu, Z.; Miao, Z.; Huang, X.; Sun, Z.; Xu, H.; Wang, Z., The Clinical Significance and Mechanisms of REG4 in Human Cancers. *Front Oncol* **2020**, *10*, 559230.
48. McLaughlin, T.; Falkowski, M.; Wang, J. J.; Zhang, S. X., Molecular Chaperone ERp29: A Potential Target for Cellular Protection in Retinal and Neurodegenerative Diseases. *Adv Exp Med Biol* **2018**, *1074*, 421-427.
49. Xu, J.; Shi, G. P., Vascular wall extracellular matrix proteins and vascular diseases. *Biochim Biophys Acta* **2014**, *1842* (11), 2106-2119.
50. Li, Y.; Song, J.; Yu, H.; Wu, S., PGE2 induces MUC2 and MUC5AC expression in human intrahepatic biliary epithelial cells via EP4/p38MAPK activation. *Annals of Hepatology* **2013**, *12* (3), 479-486.
51. Dong, L.; Lu, D.; Chen, R.; Lin, Y.; Zhu, H.; Zhang, Z.; Cai, S.; Cui, P.; Song, G.; Rao, D.; Yi, X.; Wu, Y.; Song, N.; Liu, F.; Zou, Y.; Zhang, S.; Zhang, X.; Wang, X.; Qiu, S.; Zhou, J.; Wang, S.; Zhang, X.; Shi, Y.; Figeys, D.; Ding, L.; Wang, P.; Zhang, B.; Rodriguez, H.; Gao, Q.; Gao, D.; Zhou, H.; Fan, J., Proteogenomic characterization identifies clinically relevant subgroups of intrahepatic cholangiocarcinoma. *Cancer Cell* **2022**, *40* (1), 70-87.e15.
52. Matull, W. R.; Andreola, F.; Loh, A.; Adiguzel, Z.; Deheragoda, M.; Qureshi, U.; Batra, S. K.; Swallow, D. M.; Pereira, S. P., MUC4 and MUC5AC are highly specific tumour-associated mucins in biliary tract cancer. *British Journal of Cancer* **2008**, *98* (10), 1675-1681.
53. Tian, Y.; Qiu, S.; Yang, S.; Jiang, Y.; Hu, H.; Yang, C.; Cao, J.; Chen, S.; Hao, M.; Li, H.; Zhu, J., The oncogenic role and prognostic value of PXDN in human stomach adenocarcinoma. *BMC Cancer* **2024**, *24* (1), 1463.
54. Presta, M.; Foglio, E.; Churrua Schuind, A.; Ronca, R., Long Pentraxin-3 Modulates the Angiogenic Activity of Fibroblast Growth Factor-2. *Front Immunol* **2018**, *9*, 2327.

55. Hashiguchi, S.; Tanaka, T.; Mano, R.; Kondo, S.; Kodama, S., CCN2-induced lymphangiogenesis is mediated by the integrin $\alpha\beta 5$ –ERK pathway and regulated by DUSP6. *Scientific Reports* **2022**, *12* (1), 926.
56. Ma, Y.; Chang, N.; Liu, Y.; Liu, F.; Dong, C.; Hou, L.; Qi, C.; Yang, L.; Li, L., Silencing IQGAP1 alleviates hepatic fibrogenesis via blocking bone marrow mesenchymal stromal cell recruitment to fibrotic liver. *Molecular Therapy Nucleic Acids* **2022**, *27*, 471-483.
57. Bukong, T. N.; Maurice, S. B.; Chahal, B.; Schaeffer, D. F.; Winwood, P. J., Versican: a novel modulator of hepatic fibrosis. *Laboratory Investigation* **2016**, *96* (3), 361-374.
58. Liu, Z.; Liu, H.; Xiao, L.; Liu, G.; Sun, L.; He, L., STC-1 ameliorates renal injury in diabetic nephropathy by inhibiting the expression of BNIP3 through the AMPK/SIRT3 pathway. *Laboratory Investigation* **2019**, *99* (5), 684-697.
59. Li, F.; Liu, C. S.; Wu, P.; Ling, A. S.; Pan, Q.; Li, X. N., CCT4 suppression inhibits tumor growth in hepatocellular carcinoma by interacting with Cdc20. *Chin Med J (Engl)* **2021**, *134* (22), 2721-2729.
60. Andreozzi, M.; Quintavalle, C.; Benz, D.; Quagliata, L.; Matter, M.; Calabrese, D.; Tosti, N.; Ruiz, C.; Trapani, F.; Tornillo, L.; Fusco, A.; Heim, M. H.; Ng, C. K. Y.; Pallante, P.; Terracciano, L. M.; Piscuoglio, S., HMGA1 Expression in Human Hepatocellular Carcinoma Correlates with Poor Prognosis and Promotes Tumor Growth and Migration in in vitro Models. *Neoplasia* **2016**, *18* (12), 724-731.
61. Wan, S.; Liu, X.; Sun, R.; Liu, H.; Jiang, J.; Wu, B., Activated hepatic stellate cell-derived Bmp-1 induces liver fibrosis via mediating hepatocyte epithelial-mesenchymal transition. *Cell Death & Disease* **2024**, *15* (1), 41.
62. Wang, Y.; Yan, W.; Lu, Y.; Du, J.; Tian, X.; Wu, B.; Peng, S.; Gu, B.; Cai, W.; Xiao, Y., Intestinal Reg4 deficiency confers susceptibility to high-fat diet-induced liver steatosis by increasing intestinal fat absorption in mice. *JHEP Reports* **2023**, *5* (6).
63. Chen, X. M.; O'Hara, S. P.; LaRusso, N. F., The immunobiology of cholangiocytes. *Immunol Cell Biol* **2008**, *86* (6), 497-505.
64. Zheng, M.; Tian, Z., Liver-Mediated Adaptive Immune Tolerance. *Frontiers in Immunology* **2019**, Volume 10 - 2019.
65. Guo, H.; Zhu, Q.; Yu, X.; Merugu, S. B.; Mangukiy, H. B.; Smith, N.; Li, Z.; Zhang, B.; Negi, H.; Rong, R.; Cheng, K.; Wu, Z.; Li, D., Tumor-secreted anterior gradient-2 binds to VEGF and FGF2 and enhances their activities by promoting their homodimerization. *Oncogene* **2017**, *36* (36), 5098-5109.
66. Tang, Y.; He, Y.; Li, C.; Mu, W.; Zou, Y.; Liu, C.; Qian, S.; Zhang, F.; Pan, J.; Wang, Y.; Huang, H.; Pan, D.; Yang, P.; Mei, J.; Zeng, R.; Tang, Q. Q., RPS3A positively regulates the mitochondrial function of human periaortic adipose tissue and is associated with coronary artery diseases. *Cell Discov* **2018**, *4*, 52.
67. Fang, Y.; Xie, T.; Xue, N.; Kuang, Q.; Wei, Z.; Liang, M.; Ding, X., miR-382 Contributes to Renal Tubulointerstitial Fibrosis by Downregulating HSPD1. *Oxid Med Cell Longev* **2017**, *2017*, 4708516.
68. Eelen, G.; de Zeeuw, P.; Simons, M.; Carmeliet, P., Endothelial cell metabolism in normal and diseased vasculature. *Circ Res* **2015**, *116* (7), 1231-44.

Chapter V

Conclusion Remarks and Future Perspectives

Conclusion remarks and future perspectives

A major limitation in organoid culture is the absence of both vasculature and stromal compartments, which together impair nutrient and oxygen diffusion, restrict organoid size and viability, and hinder the ability to model complex cell–cell and cell–matrix interactions under physiological or pathological conditions. Throughout this work, we addressed this limitation by establishing two key strategies to emulate in vitro tissue environments with integrated vasculature and stroma: a bottom-up approach using VUs as building blocks for the formation of robust stroma-embedded perfusable microvascular networks (Chapter III), and a peri-bile ductal fibrovascular stroma-on-chip microfluidic device integrating “fibrotic” vascularized stroma and intrahepatic cholangiocyte organoids (ICOs) (Chapter IV).

The first part of this thesis introduced vascular units (VUs), microtissues composed of endothelial progenitor cells and organ-specific intestinal fibroblasts, as living building blocks capable of self-assembling into extensive, lumenized capillary networks. Embedded in fibrin and incorporated into a biomimetic vessel-on-chip (VoC), VUs were shown to inosculate with pre-formed vascular channels and become perfused, thereby establishing interconnected microvascular beds. These results validated the feasibility of VUs to bridge perfusion channels and to support a vascularized stromal compartment capable of direct interacting with multiple adult stem cell-derived intestinal organoids (IOs). This work underscores the potential of VUs as a modular and scalable platform for applications in regenerative medicine and the engineering of vascularized microphysiological in vitro systems. Their adaptability to diverse tissue/organ contexts (through tunable cellular composition) establishes VUs as a promising strategy for standardized vascularization of complex 3D tissue models. Yet, further optimization could strengthen VU-based systems.

In our studies, we focused on culturing VUs in well-established pro-angiogenic hydrogels – specifically fibrin-based formulations, which offer a significant improvement over Matrigel in terms of reproducibility. However, while fibrin-laminin hydrogels provided adequate support for both vascular network formation and organoid culture, their limited stability (typically lasting no more than two weeks in culture) remains a significant constraint for long-term studies. One promising avenue would be the use of hybrid hydrogels that combine fibrin with more mechanically stable and tuneable hydrogels, such as alginate for example^[1]. These formulations should not only support angiogenic sprouting and vascular morphogenesis but also offer improved matrix durability over time. This would enable the maintenance of the vascular structures for longer periods and, at the same time, allow for fine-tuning of mechanical properties to better mimic both healthy and diseased tissue microenvironments.

The development of standardized assays to evaluate VU functionality – such as vascular network formation, lumen integrity, and responsiveness to angiogenic stimuli, remains an area with considerable room for advancement. Our vessel-on-chip (VoC) platform, particularly when combined with an advanced high-throughput bioimaging system, contributed to addressing this gap by enabling real-time assessment of VU integration, network connectivity, and functional perfusion under biomimetic conditions.

However, integrating VUs into microfluidic chambers presented certain challenges, particularly regarding their controlled positioning within the system in order to avoid unintended fusion, sedimentation, and uneven spacing, while ensuring the formation of a well-connected vascular network during sprouting. To address these limitations, the development of modular microfluidic platforms with integrated positioning features – such as physical micropatterns, hydrogel anchors, or guided loading channels, may offer a viable solution. Alternatively, controlled deposition techniques using microinjection systems or robotic dispensers could enable the precise placement of VUs within the chamber. Such strategies would support the spatial organization of multiple VUs, facilitating the study of inter-VU interactions and promoting consistent vascular network formation, including reliable inosculation with perfusion channels.

Once established, the inosculation of VUs with perfusion channels must be functionally validated to confirm that perfusate can effectively infiltrate and flow through the VU-derived vascular network. In Chapter III, we demonstrated successful inosculation and perfusion by introducing fluorescent microbeads into the Vessel-on-Chip (VoC) channels. Using 3D confocal reconstructions, we confirmed the presence of microbeads within the lumens of VU-derived capillaries, providing direct evidence of functional flow. However, both the flow dynamics and perfusion assessment methods leave room for optimization. As further explored in Chapter IV, redesigning the chip to include medium reservoir compartments enabled gravity-driven flow, which minimized bubble formation—commonly caused by pump tubing issues—and simplified overall handling. In addition, replacing microbeads with smaller fluorescent tracers such as labelled dextrans (70–500 kDa) under constant gravity flow could enhance perfusion analysis, as microbeads frequently aggregate and display suboptimal flow behaviour. This alternative approach would improve fluid dynamics, enabling more consistent perfusate infiltration from arteriole-like channels into the finer VU capillaries, thereby increasing the sensitivity and reliability of perfusion-related readouts.

In Chapter III, we also demonstrated that the vascularized stroma-on-chip developed in conjunction with and around intestinal organoids, creating a supportive environment for cellular crosstalk and perfusion. While this provided proof-of-concept for the use of VUs as modular

units to generate vascularized stroma in organoid cultures, the specific influence of vascular and stromal cell interactions on organoid phenotype and function was not investigated in this study. As a future direction, it would be valuable to examine how vascularization affects organoid cellular differentiation and functional maturation, using omics-based approaches – such as transcriptomics and/or proteomics, as primary analytical tools.

The second study presented in this thesis focused on the development of a peri-bile ductal fibrovascular stroma-on-chip platform. Intrahepatic cholangiocyte organoids (ICOs) were selected as the epithelial component due to their well-characterized progenitor-like profile and their relevance in liver fibrosis, where reactive cholangiocytes are known to engage in crosstalk with both stromal and vascular cells. ICOs possess the capacity to differentiate toward hepatocyte- and cholangiocyte-like lineages, making them a valuable model for studying epithelial plasticity in fibrogenic settings. Their functional assessment is relatively straightforward, supported by a range of established assays and well-defined maturation marker panels available in the literature. Moreover, few studies have explored cellular crosstalk within in vitro systems that recapitulate the peribiliary vascular plexus (PVP), positioning ICOs as a relevant yet underutilized model for investigating multicellular dynamics in biliary disease contexts.

In this study, we first identified fibrin-laminin (FIB-LAM) hydrogels as a superior matrix environment for ICO culture and maturation when compared to traditional Matrigel-based systems. FIB-LAM promoted typical cystic morphologies, improved lumen formation, and supported early differentiation features, establishing a defined matrix condition more amenable to controlled co-culture systems. Nevertheless, FIB-LAM gels were not suitable to sustain long-term organoids culture, especially when submitted to differentiation protocols as hepatic organoids tend to enhance matrix degradation and MMPs expression^[2, 3]. In future research, the use of hydrogels with controllable degradation properties will be essential for supporting long-term 3D cultures, particularly of highly metabolically active organoids, which exert significant mechanical and enzymatic pressure on their surrounding matrix during growth and maturation.

Building on this foundation, we then integrated ICOs with endothelial colony-forming cells (ECFCs) and hepatic stellate cells (HSCs) within a microfluidic platform to model multicellular crosstalk in a fibrogenic, vascularized microenvironment. This allowed us to investigate how direct interactions among epithelial, stromal, and vascular compartments influence both organoid behaviour and the structural organization of the surrounding stromal-vascular network.

Leveraging the highly customizable design of the VoC described in Chapter III, the chip was adapted to incorporate a gravity-driven perfusion system with two lateral medium reservoir chambers, which is much simpler to operate and maintain than pump-driven ones. To expand the vascularized surface area and promote interactions with multiple organoids, three parallel channels were cast and seeded with ECFCs and HSCs, forming the vascularized stromal compartment. VUs were deliberately excluded to streamline the set-up. As this study focused on mimicking the peribiliary vascular plexus (PVP) and investigating the impact of multicellular crosstalk on ICO behaviour, the device architecture was intentionally simplified to emphasize organoid-related functional readouts.

When added to the chip channels, ECFCs and HSCs formed a dense and perfusable fibro-vascular network. It is known that primary HSCs undergo spontaneous activation when expanded on plastic surfaces, losing their vitamin A-containing lipid droplets and acquiring a pro-fibrotic like profile^[4]. We assessed the activation state of the primary HSCs used in our model and found that they exhibited elevated α SMA expression compared to other non-activated cell types, but lower levels than HSCs activated with TGF β of CAFs. This suggests that the cells were in an early activation stage, likely induced by mechanical cues from the 2D expansion culture environment. While suitable for fibrotic modelling, future studies aiming to mimic a homeostatic liver niche should consider using quiescent HSCs and including other non-parenchymal cells such as Kupffer cells or MSCs, to improve physiological relevance.

The inclusion of ICOs in the chip, alongside ECFCs and HSCs, inhibited early angiogenic activity and HSC outgrowth, and led to compromised vascular integrity. This effect is likely mediated by paracrine signalling between ICOs, ECFCs, and pre-activated HSCs, and consequent modulation of HSCs' contractile phenotype. These findings highlight the utility of our model for dissecting complex multicellular interactions relevant to liver disease pathophysiology.

The LC–MS/MS proteomics analysis of secretomes from chips containing both ICOs and a fibrovascular stroma revealed a distinctive pro-fibrotic and inflammatory profile, compared to controls. This data supports a model in which reactive ICOs initiate a cyclic, self-sustaining crosstalk with hepatic HSCs, leading to mutual activation and amplification of fibrogenic signalling. The resulting microenvironment is characterized by increased inflammatory mediators, enhanced cell death, and contractile activity, ultimately compromising vascular structure and function.

Ongoing work will aim to dissect the molecular drivers of this feedback loop through RNA-seq analysis, supported by bioinformatics tools to correlate transcriptomic and proteomic data and

help confirm key observations. Additionally, evaluating targeted interventions – such as cytokine inhibitors and anti-fibrotic compounds, that interfere with cholangiocyte-induced HSC activation will be critical for validating the model's relevance to fibrotic disease progression.

Surprisingly, immunoglobulin proteins (typically associated with B cells) were detected in the ICO secretome, even after excluding serum-derived contaminants from the analysis. While the biological relevance of this observation remains to be fully understood, this warrants further investigation as it may reflect an underappreciated aspect of epithelial immune mimicry or reactive secretory behaviour in fibrogenic settings.

Overall, the engineered platforms developed in this thesis offer versatile and physiologically relevant tools for advancing in vitro organoid models. By integrating vascular, stromal, and epithelial compartments within customizable microfluidic systems, this work enhances our ability to model complex tissue microenvironments with greater fidelity. Although challenges such as long-term perfusion stability, immune cell integration, and high-throughput scalability remain, these models represent a significant step forward in reducing reliance on animal models and enabling more predictive, human-relevant preclinical testing.

References

1. Barros da Silva, P.; Zhao, X.; Bidarra, S. J.; Nascimento, D. S.; LaLone, V.; Lourenço, B. N.; Paredes, J.; Stevens, M. M.; Barrias, C. C., Tunable Hybrid Hydrogels of Alginate and Cell-Derived dECM to Study the Impact of Matrix Alterations on Epithelial-to-Mesenchymal Transition. *Advanced Healthcare Materials* **2024**, *13* (29), 2401032.
2. Li, H.; Li, J.; Wang, T.; Sun, K.; Huang, G.; Cao, Y.; Wu, F.; Xu, A., Hepatobiliary organoids differentiated from hiPSCs relieve cholestasis-induced liver fibrosis in nonhuman primates. *International Journal of Biological Sciences* **2024**, *20* (4), 1160-1179.
3. Li, K.; He, Y.; Jin, X.; Jin, K.; Qian, J., Reproducible extracellular matrices for tumor organoid culture: challenges and opportunities. *Journal of Translational Medicine* **2025**, *23* (1), 497.
4. Gandhi, C. R., Hepatic stellate cell activation and pro-fibrogenic signals. *Journal of Hepatology* **2017**, *67* (5), 1104-1105.

