Thrombosis-on-a-Chip

Microfluidic Cell Culture Models
with Whole Blood Perfusion

Hugo J. Albers

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Table of Contents

Chapter 1. Introduction & Thesis Outline.....	13
Introduction.....	14
Organ-on-Chip Models	16
Possible Improvements and Challenges	17
Aim of the Thesis.....	19
Thesis Outline.....	19
References	20
 Chapter 2. Advanced <i>in vitro</i> Models of Vascular Biology: Human Induced Pluripotent Stem Cells and Organ-on-Chip Technology	25
Introduction.....	27
Human iPSC-Derived Vascular Cells	29
Human iPSC-Derived Endothelial Cells	30
Human iPSC-Derived Vascular Smooth Muscle Cells and Pericytes	31
Patient hiPSC-Derived Vascular Cells.....	33
Organ-on-Chip Technology for Vascular Biology.....	33
Vascular Barrier Function in Chips.....	34
Regulation of Blood Flow, Hemostasis and Thrombosis.....	36
Vasculogenesis, Angiogenesis and Vessel Morphology	37
Integration of iPSC-Derived Vascular Cells in Organ-on-Chip Systems	38
Future Perspectives	39
Characterization and Quality Control of hiPSC-Derived Vascular Cells...	39
Standardization and Robustness of Organ-on-Chip Models	40
Advanced <i>in vitro</i> Patient-Specific Disease Modeling.....	41
Advanced <i>in vitro</i> Models in Drug Development.....	42
Outlook	43
References	44

Chapter 3. Mimicking Arterial Thrombosis in a 3D-Printed Microfluidic *in vitro* Vascular Model Based on Computed Tomography Angiography Data 55

Introduction	57
Materials & Methods	59
Coronary Artery 3D Models	59
Microfluidic Device Design and Fabrication	59
Endothelial Cell Culture and Seeding	62
Staining, Fluorescence Microscopy and Confocal Microscopy	62
Blood Perfusion.....	62
Computational Fluid Dynamics	63
Results & Discussion.....	64
Fabrication of Microfluidic Chips with 3D Blood Vessel Geometries.....	64
Endothelialisation of Microfluidic Chips with 3D Model Vessels.....	65
Computational Fluid Dynamics on the 3D Vessel Geometries	66
Thrombosis-on-a-Chip.....	68
Conclusions	70
References.....	71

Chapter 4. Automated Analysis of Platelet Aggregation on Cultured Endothelium in a Microfluidic Chip Perfused with Human Whole Blood75

Introduction	77
Materials & Methods	79
Microfluidic Device Fabrication	79
Endothelial Cell Seeding and Introduction of Inflammatory Cytokines	79
Whole Blood Perfusion	80
Determination of an ROI and Triangle Thresholding	81
Manual Identification of Platelet Aggregates and Statistics	81

Computational Fluid Dynamics	81
Results	82
Microfluidic Device and Introduction of Data	82
Automated Threshold Using the Triangle Method	83
Comparison of Binary Output Using the Triangle Method with the Manual Method.....	87
Discussion	90
Conclusions	91
Supplementary Materials.....	91
References	92
Chapter 5. Human Induced Pluripotent Stem Cell-Derived Endothelial Cells in Microfluidic Channels Perfused with Human Whole Blood Enable Systematic Studies of Platelet Aggregation	95
Introduction.....	97
Materials & Methods	98
Chip Fabrication	98
Cell Culture	99
Whole Blood Perfusion.....	100
Fluorescent Labeling and Immunostaining.....	100
Microscopy and Image Analysis	101
Statistics	102
Results	102
Human Endothelial Cells Cultured in Microfluidic Channels Form Monolayers and can be Stimulated with Inflammatory Cytokines.....	102
Platelet Aggregates Form on Inflamed Endothelium in the Microfluidic Channels upon Whole Blood Perfusion at Arterial Shear Rates.....	104

Platelet Aggregation on hiPSC-ECs in Microfluidic Channels can be Inhibited by Pre-Treatment of Whole Blood with a Clinical Anti-Platelet Compound.....	107
Discussion	108
References.....	110
 Chapter 6. Local Stimulation of Endothelium with Inflammatory Factors in 3D-Printed Microfluidic Channels	117
Introduction	119
Materials & Methods	120
3D-Printing.....	120
Chip Fabrication	121
Cell Seeding.....	122
Human Whole Blood Perfusion.....	123
Results & Discussion.....	123
Mold and Chip Fabrication	123
Incorporation of Endothelial Cells	126
Whole Blood Perfusion	128
Conclusions.....	130
Recommendations.....	130
References.....	131
 Chapter 7. Summary & Outlook	135
Summary	136
Outlook & Future Work	137
Human Induced Pluripotent Stem Cell-Derived Cells.....	137
Microfluidic Channel Architectures.....	138
Applications	139

References	140
Appendix.	141
A. Supplementary Information Chapter III.....	142
B. Supplementary Information Chapter IV	148
C. Supplementary Information Chapter V.....	151
D. Supplementary Information Chapter VI	153
E. Cilostazol as Antiplatelet Therapeutic.....	155
References	157
F. Thrombosis-on-a-Chip for Sepsis Studies	158
References	160
 List of Publications	 161
Samenvatting.....	167
Contributions.....	169
Acknowledgements.....	171

Chapter 1

Introduction & Thesis Outline

1.1. Introduction

Cardiovascular diseases (CVDs) are the number one cause of death worldwide (31% in 2016) [1]. The majority of CVD-related deaths are due to heart attacks or stroke (85% of all CVD-related deaths), which are often instigated by thrombosis, *i.e.* the formation of blood clots inside of blood vessels. Therefore, both the academic world and industry seek to better understand, prevent and treat CVDs and thrombosis. However, the number of CVD-related deaths are still rising. Furthermore, the number of therapeutics in the drug development pipeline has stagnated and is disproportionately lower in comparison to diseases with a lower burden on society, *e.g.* cancer or infectious diseases [2]. The development of novel therapeutics is a long and expensive process, currently taking twelve to fifteen years and over one billion euros per new drug [2,3]. The rise in CVD-related deaths, the stagnated number of therapeutics in the drug development pipeline and high costs are a cause for worry and asks for a more efficient drug development process.

Drug development is executed in three stages followed by regulatory approval of a safe new therapeutic (Figure 1) [4]. In the first and second phases a large number of compounds are discovered and screened. These pre-clinical trials are executed using *in vitro* and animal models. In the third phase a series of human studies are conducted to investigate safety and efficacy.

Current pre-clinical *in vitro* studies are often conducted using high throughput techniques. For example, human cells can be cultured in microplate wells, which are polystyrene arrays of small culture chambers. In these wells growth factors, nutrients and compounds of interest can be introduced to investigate the cellular response. Unfortunately, human cells cultured in simple two-dimensional wells do not fully respond as they would in their natural environment [5,6]. Alternatively, various animal models can be used that are capable of mimicking complex CVDs, even CVDs that would normally not occur in these animals [7]. However, results from animal studies do not always translate into similar results in humans [8–10]. Furthermore, the use of animal models is expensive, ethically questionable and, if these models have a poor predictive value, unnecessary [11]. Therefore, efforts should be made to replace, reduce and refine (the three Rs) animal models.

Despite the extensive use of pre-clinical models in drug development, a 80% of all drug candidates still fail in the clinical trials, often due to a lack of efficacy or unexpected side-effects [12]. Regardless of the reason for termination, the high rates of late stage failure indicate a poor predictive power of the *in vitro* and animal models. Therefore, pre-clinical models that can reliably mimic human physiology and disease pathophysiology to predict the effects of therapeutics in humans are needed.

Figure 1. Schematic of the drug development pipeline. Phase 1 and 2 focus on the discovery and screening of a large number of compounds in simple *in vitro* models and using animal models. Only a small number of compounds move to phase 3, where a series of clinical trials are conducted hopefully followed by regulatory approval.

1.1.1. Organ-on-Chip Models

There is a need for models in which human cells or tissues are cultured in a micro-environment that emulates the physiological situation in such a way that the cultured tissues behave in a more realistic manner. Two promising solutions have emerged in the past decades: organoids and organ-on-a-chip devices.

Organoid cultures allow for the self-assembled generation of complex and three-dimensional (3D) co-cultures showing organ-like functionality [13]. Although these models are promising, research in CVD often requires active perfusion to mimic blood flow, which is not compatible with organoid cultures [14].

Alternatively, organ-on-a-chip devices could be the answer to the need for technology that can mimic human response [8]. Organ-on-a-chip technology relies on microfluidic technology to provide a realistic environment for human cells to be cultured. Furthermore, patient-specific cells can be incorporated which allows for a personalized approach [15]. The use of patient-specific cells could improve the prediction of efficacy and toxicity during pre-clinical studies [9]. Furthermore, it could lead to the implementation of a personalized medicine approach where a specific drug combination and dose is tailored for each patient [9]. These microfluidic organ-on-chip devices have micrometer-size channels in which human cells can be cultured. Furthermore, similar biochemical cues can be applied as in standard *in vitro* tests but with the added benefit of control over physical properties like: fluid flow and disease-specific geometries [5].

Fluid flow is an important parameter when emulating human physiology in a model, especially in the case of CVDs, where whole blood perfusion plays a pivotal role. This is particularly true when studying thrombosis, where the interplay of activated endothelium, coagulation factors, leukocytes and platelets is extremely important in this pathological process [33]. Others have reported on using whole blood perfusion to investigate platelet biology in parallel plate flow chambers. These chambers were patterned with extracellular matrix patches to investigate adhesion of coagulation factors and platelets [16–23]. Alternatively, various vasculature-on-chip models have been introduced that incorporate human endothelium [24–32]. Naturally a model that combines the culture of human endothelium and the perfusion of human whole blood will capture more aspects of the interplay of factors in thrombosis than models that include only whole blood and extracellular matrix.

Microfluidic organ-on-a-chip devices used for thrombosis studies, appropriately coined thrombosis-on-a-chip devices, are found with various geometries. Ranging from simple yet reproducible rectangular cross-section channels, fabricated using moulds patterned with photolithography [25,29], to circular cross-section channels either 3D-bioprinted [30] or patterned with optical fibers [26]. These thrombosis-on-a-chip devices have been applied to study the immediate effect of different geometries on blood clot formation, by investigating healthy and stenotic geometries and even aneurysms [26,28,29].

Besides using disease-specific geometries, various biochemical cues, *e.g.* inflammatory cytokines, endotoxins and therapeutics, have been investigated in thrombosis-on-a-chip devices. By introducing inflammatory cytokines, a pro-thrombotic state was induced by activating the cultured endothelium [25,28]. Furthermore, pulmonary thrombosis was investigated by emulating the complex interplay between lung epithelium and endothelium. Lung epithelium was exposed to an endotoxin resulting in a pro-thrombotic response in the endothelium [34]. While directly exposing the endothelium to the endotoxin did not result in a pro-thrombotic response. Recently Barrile et al. reported on investigating a novel therapeutic for autoimmune diseases that was terminated in clinical trials [24]. The thrombosis-on-a-chip device was able to elucidate the mechanic causing pro-thrombotic events proving that the organ-on-chip can be used to study drug toxicity.

1.1.2. Possible Improvements and Challenges

Previously introduced vasculature-on-chip devices used for thrombosis studies seem promising and were even used to investigate why a drug had failed during clinical trials. However, various improvements can still be made to increase the realism of these thrombosis-on-a-chip devices such as introducing realistic disease-specific geometries, culture of person-specific endothelial cells and automated data analysis.

The chosen geometries influence the fluid dynamical properties of the device, *e.g.* microfluidic channels with rectangular cross-sections inevitably display low flow zones near the corners [35]. When analyzing only a constant shear rate, these edge-effects can be ignored by focusing only on a specific zone in the middle of the device. However, for applications with complex fluid dynamical patterns, *e.g.* a stenosis or aneurysm, a square or rectangular channel

geometry is very undesirable. Designing and fabricating a microfluidic system based on human computed tomography angiography data has not been done before and could potentially be used to investigate arterial thrombosis induced by stenotic geometries. Furthermore, by adding a secondary channel and a semi-permeable barrier various inflammatory cytokines can be introduced to locally induce inflammation.

Another important parameter is the source of the vascular tissue used in the microfluidic models. Incorporation of human primary endothelial cells is not always the best option. Currently, human umbilical vein endothelial cells (HUVECs) are often used in vascular models, since the umbilical vein used to harvest the HUVECs is regarded as surgical waste and thus is easy to come by. However, cells from multiple donors are often combined resulting in a batch-to-batch variability [36]. Alternatively, pluripotent stem cells could be used as a source of endothelial cells. Since the discovery of human induced pluripotent stem cells (hiPSCs) [37], robust protocols for differentiating hiPSCs into arterial lineage have been reported [38,39]. To investigate whether these human induced pluripotent stem cell-derived endothelial cells (hiPSC-ECs) can be used in assays others have compared the inflammatory properties of hiPSC-ECs to primary ECs, showing a more moderate response to inflammatory cytokines [40]. Furthermore, hiPSC-ECs have been cultured in patterned extracellular matrix lumens in microfluidic devices [41]. Incorporating these hiPSC-ECs in microfluidic channels opens up opportunity to fabricate personalized thrombosis-on-a-chip devices. To the best of the author's knowledge no report of hiPSC-ECs in thrombosis-on-a-chip devices exist.

Finally, as the complexity of thrombosis-on-a-chip devices increases, the need for a robust methodology of analyzing data arises. The expected platelet aggregation in these thrombosis-on-a-chip devices depends on the pre-treatment of the endothelium. Untreated endothelium will not induce platelet aggregation, while activated endothelium will result in 3D platelet aggregates. The heterogeneous nature of the expected data requires a unique data analysis strategy.

1.1.3. Aim of the Thesis

The aim of this PhD project was to develop new thrombosis-on-a-chip devices that can more realistically mimic human physiology and disease pathophysiology. To this end, the aforementioned possible improvements were investigated with a focus on: realistic disease-specific geometries, the incorporation of hiPSC-ECs, a robust data analysis methodology and an approach to locally activate endothelium. These improvements to thrombosis-on-a-chip devices will bring them closer to being used routinely as pre-clinical models in the drug development pipeline. Furthermore, by incorporating person-specific cells, the models could potentially be utilized for future studies in personalized medicine. The proposed devices and solutions are briefly introduced in the thesis outline below.

1.2. Thesis Outline

This thesis consists of seven chapters, starting with a brief introduction in **Chapter I**. In **Chapter II** an overview of the use of hiPSC-derived cells for *in vitro* models of vasculature is given. Furthermore, an overview of various organ-on-a-chip devices and a possible implementation of hiPSC-derived cells in organ-on-a-chip devices is discussed. Four experimental chapters are included in this thesis. A 3D-printed thrombosis-on-a-chip device based on computed tomography angiography data is introduced in **Chapter III**. The effect of stenotic geometries on platelet aggregation in microfluidic channels lined with primary endothelium and perfused with human whole blood is investigated. **Chapter IV** introduces an automated methodology that can be used to process fluorescence microscopy data of whole blood perfusion in microfluidic channels lined with endothelial cells to measure platelet coverage and platelet aggregate size distributions. This methodology is utilized to quantitatively compare an often used source of endothelial cells human umbilical vein endothelial cells with human induced pluripotent stem cell-derived endothelial cells in **Chapter V**. Furthermore, a proof-of-principle study is discussed where an antiplatelet therapeutic is added to the human whole blood prior to perfusion in a microfluidic channel lined with inflamed hiPSC-ECs. The final experimental **Chapter VI** discusses the fabrication and characterization of a two-channel

microfluidic device used to locally inflame endothelial cells for thrombosis studies. **Chapter VII** summarizes the results and provides a brief outlook.

1.3. References

1. WHO Fact Sheet - Cardiovascular diseases Available online: <https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-cvds> (accessed on Jul 15, 2019).
2. Fordyce, C.B.; Roe, M.T.; Ahmad, T.; Libby, P.; Borer, J.S.; Hiatt, W.R.; Bristow, M.R.; Packer, M.; Wasserman, S.M.; Braunstein, N.; et al. Cardiovascular Drug Development: Is it Dead or Just Hibernating? *J. Am. Coll. Cardiol.* **2015**, *65*, 1567–1582.
3. Hughes, J.P.; Rees, S.; Kalindjian, S.B.; Philpott, K.L. Principles of early drug discovery. *Br. J. Pharmacol.* **2011**, *162*, 1239–49.
4. FDA The Drug Development Process Available online: <https://www.fda.gov/patients/learn-about-drug-and-device-approvals/drug-development-process> (accessed on Nov 25, 2019).
5. Meer, A.D. van der; Berg, A. van den Organs-on-chips: breaking the in vitro impasse. *Integr. Biol.* **2012**, *4*, 461.
6. van de Stolpe, A.; den Toonder, J. Workshop meeting report Organs-on-Chips: human disease models. *Lab Chip* **2013**, *13*, 3449.
7. Zaragoza, C.; Gomez-Guerrero, C.; Martin-Ventura, J.L.; Blanco-Colio, L.; Lavin, B.; Mallavia, B.; Tarin, C.; Mas, S.; Ortiz, A.; Egido, J. Animal models of cardiovascular diseases. *J. Biomed. Biotechnol.* **2011**, *2011*, 497841.
8. Bhatia, S.N.; Ingber, D.E. Microfluidic organs-on-chips. *Nat. Biotechnol.* **2014**, *32*, 760–772.
9. Esch, E.W.; Bahinski, A.; Huh, D. Organs-on-chips at the frontiers of drug discovery. *Nat. Rev. Drug Discov.* **2015**, *14*, 248–60.
10. Mak, I.W.; Evaniew, N.; Ghert, M. Lost in translation: animal models and clinical trials in cancer treatment. *Am. J. Transl. Res.* **2014**, *6*, 114–8.
11. Huh, D.; Torisawa, Y.; Hamilton, G.A.; Kim, H.J.; Ingber, D.E. Microengineered physiological biomimicry: Organs-on-Chips. *Lab Chip* **2012**, *12*, 2156.
12. Fogel, D.B. Factors associated with clinical trials that fail and opportunities for improving the likelihood of success: A review. *Contemp. Clin. trials Commun.* **2018**, *11*, 156–164.
13. Sato, T.; Clevers, H. Growing self-organizing mini-guts from a single intestinal stem cell: mechanism and applications. *Science* **2013**, *340*, 1190–4.
14. Ingber, D.E. Reverse Engineering Human Pathophysiology with Organs-on-Chips. *Cell* **2016**, *164*, 1105–1109.
15. van den Berg, A.; Mummery, C.L.; Passier, R.; van der Meer, A.D. Personalised organs-on-chips: functional testing for precision medicine. *Lab*

- Chip* **2019**, *19*, 198–205.
16. Colace, T.; Falls, E.; Zheng, X.L.; Diamond, S.L. Analysis of morphology of platelet aggregates formed on collagen under laminar blood flow. *Ann. Biomed. Eng.* **2011**, *39*, 922–929.
 17. Neeves, K.B.; Onasoga, A.A.; Hansen, R.R.; Lilly, J.J.; Venckunaite, D.; Sumner, M.B.; Irish, A.T.; Brodsky, G.; Manco-Johnson, M.J.; Di Paola, J.A. Sources of Variability in Platelet Accumulation on Type 1 Fibrillar Collagen in Microfluidic Flow Assays. *PLoS One* **2013**, *8*.
 18. Colace, T. V.; Diamond, S.L. Direct observation of von Willebrand factor elongation and fiber formation on collagen during acute whole blood exposure to pathological flow. *Arterioscler. Thromb. Vasc. Biol.* **2013**, *33*, 105–113.
 19. Hansen, R.R.; Tipnis, A.A.; White-Adams, T.C.; Di Paola, J.A.; Neeves, K.B. Characterization of collagen thin films for von Willebrand factor binding and platelet adhesion. *Langmuir* **2011**, *27*, 13648–13658.
 20. Six, K.R.; Devloo, R.; Van Aelst, B.; Vandekerckhove, P.; Feys, H.B.; Compennolle, V. A Microfluidic Flow Chamber Model for Platelet Transfusion and Hemostasis Measures Platelet Deposition and Fibrin Formation in Real-time. *J. Vis. Exp.* **2017**, e55351.
 21. Van Kruchten, R.; Cosemans, J.M.E.M.; Heemskerk, J.W.M. Measurement of whole blood thrombus formation using parallel-plate flow chambers a practical guide. *Platelets* **2012**, *23*, 229–242.
 22. de Witt, S.M.; Swieringa, F.; Cavill, R.; Lamers, M.M.E.; van Kruchten, R.; Mastenbroek, T.; Baaten, C.; Coort, S.; Pugh, N.; Schulz, A.; et al. Identification of platelet function defects by multi-parameter assessment of thrombus formation. *Nat. Commun.* **2014**, *5*, 4257.
 23. Matsui, H.; Sugimoto, M.; Mizuno, T.; Tsuji, S.; Miyata, S.; Matsuda, M.; Yoshioka, A. Distinct and concerted functions of von Willebrand factor and fibrinogen in mural thrombus growth under high shear flow. *Blood* **2002**, *100*, 3604–10.
 24. Barrile, R.; van der Meer, A.D.; Park, H.; Fraser, J.P.; Simic, D.; Teng, F.; Conegliano, D.; Nguyen, J.; Jain, A.; Zhou, M.; et al. Organ-on-Chip Recapitulates Thrombosis Induced by an anti-CD154 Monoclonal Antibody: Translational Potential of Advanced Microengineered Systems. *Clin. Pharmacol. Ther.* **2018**, *104*, 1240–1248.
 25. Jain, A.; van der Meer, A.D.; Papa, A.L.; Barrile, R.; Lai, A.; Schlechter, B.L.; Otieno, M.A.; Loudon, C.S.; Hamilton, G.A.; Michelson, A.D.; et al. Assessment of whole blood thrombosis in a microfluidic device lined by fixed human endothelium. *Biomed. Microdevices* **2016**, *18*, 1–7.
 26. Mannino, R.G.; Myers, D.R.; Ahn, B.; Wang, Y.; Margo Rollins; Gole, H.; Lin, A.S.; Guldberg, R.E.; Giddens, D.P.; Timmins, L.H.; et al. “Do-it-yourself in vitro vasculature that recapitulates in vivo geometries for investigating

- endothelial-blood cell interactions". *Sci. Rep.* **2015**, 5, 12401.
27. Mathur, T.; Singh, K.A.; R. Pandian, N.K.; Tsai, S.-H.; Hein, T.W.; Gaharwar, A.K.; Flanagan, J.M.; Jain, A. Organ-on-chips made of blood: endothelial progenitor cells from blood reconstitute vascular thromboinflammation in vessel-chips. *Lab Chip* **2019**, 19, 2500–2511.
28. Menon, N.V.; Tay, H.M.; Wee, S.N.; Li, K.H.H.; Hou, H.W. Micro-engineered perfusable 3D vasculatures for cardiovascular diseases. *Lab Chip* **2017**, 17, 2960–2968.
29. Westein, E.; van der Meer, A.D.; Kuijpers, M.J.E.; Frimat, J.-P.; van den Berg, A.; Heemskerk, J.W.M. Atherosclerotic geometries exacerbate pathological thrombus formation poststenosis in a von Willebrand factor-dependent manner. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, 110, 1357–62.
30. Zhang, Y.S.; Davoudi, F.; Walch, P.; Manbachi, A.; Luo, X.; Dell'Erba, V.; Miri, A.K.; Albadawi, H.; Arneri, A.; Li, X.; et al. Bioprinted thrombosis-on-a-chip. *Lab Chip* **2016**, 16, 4097–4105.
31. Zheng, Y.; Chen, J.; Craven, M.; Choi, N.W.; Totorica, S.; Diaz-Santana, A.; Kermani, P.; Hempstead, B.; Fischbach-Teschl, C.; López, J.A.; et al. In vitro microvessels for the study of angiogenesis and thrombosis. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, 109, 9342–7.
32. Kolesky, D.B.; Truby, R.L.; Gladman, A.S.; Busbee, T.A.; Homan, K.A.; Lewis, J.A. 3D bioprinting of vascularized, heterogeneous cell-laden tissue constructs. *Adv. Mater.* **2014**, 26.
33. Coenen, D.M.; Mastenbroek, T.G.; Cosemans, J.M.E.M. Platelet interaction with activated endothelium: mechanistic insights from microfluidics. *Blood* **2017**, 130, 2819–2828.
34. Jain, A.; Barrile, R.; van der Meer, A.D.; Mammoto, A.; Mammoto, T.; De Ceunynck, K.; Aisiku, O.; Otieno, M.A.; Loudon, C.S.; Hamilton, G.A.; et al. Primary Human Lung Alveolus-on-a-chip Model of Intravascular Thrombosis for Assessment of Therapeutics. *Clin. Pharmacol. Ther.* **2018**, 103, 332–340.
35. van der Helm, M.W.; van der Meer, A.D.; Eijkel, J.C.T.; van den Berg, A.; Segerink, L.I. Microfluidic organ-on-chip technology for blood-brain barrier research. *Tissue Barriers* **2016**, 4.
36. Cochrane, A.; Albers, H.J.; Passier, R.; Mummery, C.L.; van den Berg, A.; Orlova, V. V.; van der Meer, A.D. Advanced in vitro models of vascular biology: Human induced pluripotent stem cells and organ-on-chip technology. *Adv. Drug Deliv. Rev.* **2018**.
37. Takahashi, K.; Tanabe, K.; Ohnuki, M.; Narita, M.; Ichisaka, T.; Tomoda, K.; Yamanaka, S. Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. *Cell* **2007**, 107, 861–872.
38. 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. *Nat. Protoc.* **2014**, 9, 1514–1531.
39. Orlova, V. V.; Drabsch, Y.; Freund, C.; Petrus-Reurer, S.; Van Den Hil, F.E.; Muenthaisong, S.; Ten Dijke, P.; Mummery, C.L. Functionality of endothelial cells and pericytes from human pluripotent stem cells demonstrated in cultured vascular plexus and zebrafish xenografts. *Arterioscler. Thromb. Vasc. Biol.* **2014**, 34, 177–186.
40. Halaidych, O. V.; Freund, C.; van den Hil, F.; Salvatori, D.C.F.; Riminucci, M.; Mummery, C.L.; Orlova, V. V. Inflammatory Responses and Barrier Function of Endothelial Cells Derived from Human Induced Pluripotent Stem Cells. *Stem Cell Reports* **2018**, 10, 1642–1656.
41. de Graaf, M.N.S.; Cochrane, A.; van den Hil, F.E.; Buijsman, W.; van der Meer, A.D.; van den Berg, A.; Mummery, C.L.; Orlova, V. V. Scalable microphysiological system to model three-dimensional blood vessels. *APL Bioeng.* **2019**, 3, 026105.

Chapter 2

Advanced *in vitro* Models of Vascular Biology: Human Induced Pluripotent Stem Cells and Organ-on-Chip Technology

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Abstract

The vascular system is one of the first to develop during embryogenesis and is essential for all organs and tissues in our body to develop and function. It has many essential roles including controlling the absorption, distribution and excretion of compounds and therefore determines the pharmacokinetics of drugs and therapeutics. Vascular homeostasis is under tight physiological control which is essential for maintaining tissues in a healthy state. Consequently, disruption of vascular homeostasis plays an integral role in many disease processes, making cells of the vessel wall attractive targets for therapeutic intervention. Experimental models of blood vessels can therefore contribute significantly to drug development and aid in predicting the biological effects of new drug entities. The increasing availability of human induced pluripotent stem cells (hiPSC) derived from healthy individuals and patients have accelerated advances in developing experimental *in vitro* models of the vasculature: human endothelial cells (ECs), pericytes and vascular smooth muscle cells (VSMCs), can now be generated with high efficiency from hiPSC and used in ‘microfluidic chips’ (also known as ‘organ-on-chip’ technology) as a basis for *in vitro* models of blood vessels. These near physiological scaffolds allow the controlled integration of fluid flow and three-dimensional (3D) co-cultures with perivascular cells to mimic tissue- or organ-level physiology and dysfunction *in vitro*. Here, we review recent multidisciplinary developments in these advanced experimental models of blood vessels that combine hiPSC with microfluidic organ-on-chip technology. We provide examples of their utility in various research areas and discuss steps necessary for further integration in biomedical applications so that they can contribute effectively to the evaluation and development of new drugs and other therapeutics as well as personalized (patient-specific) treatments.

2.1. Introduction

Blood vessels have a primary role in promoting the proper exchange of oxygen and nutrients in tissues across the body. In addition, the vasculature also plays key roles in the regulation of blood pressure via maintenance of vascular tone, the response to injury by regulating inflammation and hemostasis, and active transport of compounds, including therapeutics, into and out of organs. Importantly, even though the vascular system consists of a single continuous network, there are great differences between blood vessels depending on whether they are part of the arterial, microcirculatory or venous vascular beds, and the specific organ or tissue with which they interact. Given their wide array of biological functions and presence throughout the body, it is no surprise that blood vessels play an integral role in many diseases. Some diseases are primarily of a vascular nature such as coronary artery disease [1] and brain aneurysms [2], while other diseases, like diabetes and cancer, have mechanisms or complications that are directly or indirectly related to vascular dysfunction [3,4].

Experimental models of blood vessels are of great importance for understanding vascular physiology and pathophysiology. They are already being used to provide new insights into vascular function, to elucidate details of molecular signaling pathways as well as to serve as a platform for the development of new therapeutics against vascular targets. Multiple *in vivo* and *ex vivo* models of blood vessels have historically contributed significantly to our understanding of vascular biology. *In vivo* experiments on transgenic animal models such as fish, rodents, pigs and primates have been particularly informative in understanding complex vascular physiology in different tissues of interest and to determine systemic effects on the body mediated by the vasculature. Experimental *ex vivo* models are also sometimes used and based on primary human tissue, but given its limited availability (except in the case of medical “waste tissue” such as human umbilical vein), animal tissues or human cell lines have been used more extensively. However, there are many limitations of current *in vivo* and *ex vivo* models, not least ethical implications in tissue procurement and differences between animal and human vascular physiology which make it challenging to translate data from *in vivo* animal models to humans and make accurate predictions of human responses to drugs and disease. Most experimental *in vitro* cell culture models of blood vessels tend to be relatively

simple and in 2D, focusing on understanding molecular mechanisms of cellular biology rather than integrated, complex blood vessel function even though they can be based on human vascular cells. However, the key advantages of *in vitro* models are their standardized, well-controlled nature, and the possibility to systematically study human cells under a wide range of experimental conditions [5] although it is challenging in these *in vitro* studies to fully emulate the tension, flow, and cellular interactions with extracellular matrix (ECM) and supporting cells *in vivo*. Observations may thus be inaccurate representations of vascular physiology.

The most commonly used *in vitro* models of blood vessels are often grouped into three classes: monolayer, Transwell, and pseudo-capillary cell cultures. Monolayer cultures of vascular endothelial cells (ECs) or mural cells like pericytes and vascular smooth muscle cells (VSMCs) are typically used to evaluate molecular or cellular responses (morphology, proliferation, damage, migration, barrier function and contraction) to treatments with soluble factors or fluid flow. In Transwell cell cultures, vascular cell layers are grown on semi-permeable membranes which allow basic transport properties, barrier function and cell migration to be measured in the absence or presence of controlled gradients of solutes. Pseudo-capillary culture is achieved by seeding ECs on soft hydrogels and analyzing the structure of microvascular capillary-like networks that they form.

Recently, there has been a strong interest in the development of more advanced *in vitro* models of blood vessels that also allow formation of the blood vessel lumen. One driver of this research is the discovery that hiPSC can differentiate to vascular cells with fairly authentic functionality [6] (Figure 1). The second main driver is the emergence of microfluidic organ-on-chip technology [7,8] and its application in setting up smaller, yet more complex and dynamic *in vitro* models of blood vessels. Together, these developments are leading to *in vitro* models of blood vessels that capture not just the basic molecular and cell biological aspects of vascular tissue, but that also exhibit more advanced vascular physiology and morphology. It is expected that such advanced models will have a significant impact on the development of new therapeutic drugs. In the next sections, we review how hiPSC-derived vascular cells and microfluidic organ-on-chip technology are being used to model human vasculature. We then describe the efforts towards integrating hiPSC-derived cells properly into

microfluidic chips, and finally discuss the opportunities, challenges and future impact of advanced *in vitro* models of blood vessels in drug development and understanding disease.

Figure 1. Schematic summarizing methods to differentiate vascular cells from iPSCs. iPSCs are derived from human somatic cells. These can be generated from patients and furthermore, genetic editing techniques can revert the genetic mutation to create ‘healthy’ matched isogenic control cell lines. These cells are then directed towards the germ line in which the vascular cell of interest is known to derive from. Here, directed differentiation towards ECs, VSMCs or Pericytes is achieved through addition of defined factors. Vascular cell maturity can then be attained by cell co-culture, fluid flow and/or stretching and regulation of tensile strain.

2.2. Human iPSC-Derived Vascular Cells

Healthy vasculature is dependent on its key cellular components interacting and functioning in perfect synchrony. The cellular components are ECs, pericytes, VSMCs and surrounding cells in the tissues and the ECM. ECs form a monolayer which works as a selective barrier for oxygen and nutrient delivery to the tissues. In the healthy state, ECs possess anti-coagulative, anti-adhesive and anti-inflammatory properties which are critical for the proper distribution of viscous blood. Injury of the endothelium precedes many critical events that occur in atherosclerosis and inflammation, such as platelet aggregation, proliferation of VSMCs and infiltration of inflammatory cells. Pericytes and VSMCs play important roles in stabilizing EC tubes and regulating vascular tone. More recent studies indicated that pericytes in particular are very important for the induction of tissue-specific characteristics of ECs, maintenance of barrier function and regulation of inflammatory responses [9,10]. Therefore, approaches that

specifically utilize drugs that normalize or restore vascular function via targeting ECs, pericytes and/or VSMCs is potentially a route to therapy for many diseases.

To date, it has been difficult to perform drug screening on primary patient-derived vascular cells due to their limited availability and batch-to-batch variability has compromised making robust conclusions. The exciting discovery of hiPSC in 2007 [6] for which the Nobel Prize was awarded in 2012, now represents a renewable source of vascular (and other) cells without the ethical issues associated with the use of the earlier embryonic stem cells and the limited ability of adult stem cells to form cells of blood vessels.

Multiple protocols for generation of ECs, pericytes and VSMCs from hiPSC have been described and reviewed elsewhere [11,12], so will not be covered here.

2.2.1. Human iPSC-Derived Endothelial Cells

Most of the current protocols for deriving of ECs are based on 2D monolayer differentiation. These differentiation protocols all first direct hiPSC towards mesoderm specification (by adding a combination of Activin A, Bone Morphogenetic Protein-4 (BMP4), Basic Fibroblast Growth Factor (bFGF)) and induce EC specification by addition of vascular endothelial growth factor (VEGF). Other components such as transforming growth factor β (TGF β) or NOTCH inhibitors [13–15] have been added to increase EC differentiation efficiency. Alternatively, ECs can also be generated from hiPSC by simply overexpressing the core transcription factors ETV2 and GATA2 [16].

ECs display significant heterogeneity across tissue types and efforts to derive tissue-specific ECs has been the subject of many studies. Recently, a protocol for co-differentiation of ECs and cardiomyocytes from cardiac mesoderm has been developed that essentially includes induction of cardiac mesoderm followed by WNT inhibition and VEGF supplementation [17]. Importantly, WNT inhibition was also shown to support EC specification in the mouse heart [18,19]. In recent work by Thompson and colleagues [20], EC differentiation was directed towards the arterial lineage and these cells were shown to have angiogenic benefits in mouse models of myocardial infarction. The authors found that in addition to standard EC-inducing factors, such as bFGF, VEGF and the TGF β inhibitor SB431542, supplementation with the NOTCH agonist (RESV) and inositol monophosphatase inhibitor (L690) increased expression of arterial markers, such as CXCR4, EFNB2, GJA4, NRP1

and NOTCH ligands and receptors (DLL4/JAG1 and NOTCH1/4) in hiPSC-ECs and induced down-regulation of venous markers (*e.g.* NR2F2). Functionally these arterial-like ECs exhibited increased nitric oxide production, reduced leukocyte binding and improved responses to shear stress [20]. Recently, functionality of hiPSC-derived ECs was compared with primary ECs [21]. This study showed that barrier function and inflammatory responses were consistent in hiPSC-derived ECs across independent batches of cells but that they also showed some differences, such as higher barrier function and lower inflammatory responses when compared to the more widely used primary cell type, human umbilical vein endothelial cells (HUVECs). Two further studies demonstrated that exposure of hiPSC-ECs to high, arterial-like shear stress (1-2 Pa) further promoted acquisition of arterial-like characteristics and that overexpression of the RNA-binding protein QKI-5 directed EC differentiation from hiPSCs towards an arterial fate [22–24]. At present, venous and lymphatic specification of hiPSC-ECs remains a challenge with only a limited number of studies showing acquisition of some venous or lymphatic EC-associated markers; however, markers alone cannot truly confirm their identity [25]. Therefore, multi-parameter approaches that integrate vascular bed-specific hemodynamic and mechanical parameters in addition to biochemical cues are required for the development of mature and functional phenotypes in hiPSC-ECs (Figure 1) [26–28].

2.2.2. Human iPSC-Derived Vascular Smooth Muscle Cells and Pericytes

Significant progress towards differentiation of VSMCs and pericytes from hiPSC has been made recently. The primary difficulty has been that both of these cell types have different developmental origins, that are not limited to mesoderm; they can also originate from the neural crest. They are subsequently recruited to the developing EC tubes from the surrounding tissues, and their identity and functionality are largely dictated by the location in the vascular bed *in vivo*. This makes it difficult to distinguish pericytes/VSMCs from other cell types that are present in differentiating cultures due to the lack of cell type-specific surface markers. With regard to VSMC differentiation, protocols have been developed to induce these cell types from different mesoderm lineages, such as lateral plate

and cardiac (via epicardial state) mesoderm, paraxial mesoderm, ectoderm and neural crest (Figure 1) [14,29–32]. Most protocols utilize 2D monolayer differentiation methods towards the germ-lineage of interest following supplementation with Platelet-Derived Growth Factor-BB (PDGF-BB) and TGF β . Essentially, VSMCs differentiated using a combination of PDGF-BB and TGF β remain proliferative and are indistinguishable from each other based on surface marker expression. Functionally, mesoderm-derived VSMCs can be distinguished from ectoderm/neural crest cells based on their phenotypic appearances; ectoderm-derived cells having more defined epithelial cell-like morphology and lack growth inhibitory responses to TGF β . Maturation of these cells is evidenced by the acquisition of a more contractile phenotype and can be achieved by removal of PDGF-BB and either supplementation with TGF β in the presence of low or high serum concentrations, although this varies across different labs with no clear consensus at present on how best to do this. Maturation of hiPSC-derived VSMCs can be further enhanced upon administration of tensile strain (cyclic uniaxial or circumferential) [33,34] which increases the deposition of elastin protein, cell alignment and improves calcium and contractile responses. Comparison of co-cultures of primary ECs and primary VSMCs with hiPSC-ECs and hiPSC-VSMCs recently showed that in both cases, similar responses to atheroprotective or atheroprone hemodynamic cues were observed [35]. Interestingly, upon culture in atheroprotective hemodynamic conditions, genes associated with vascular maturation (specifically; EC markers KLF4, NOS3 and ASS1 and VSMC markers MYH11 and DES) were up-regulated in hiPSC-derived ECs and VSMCs. Overall, responses to stimuli and relative marker expression was quite different in hiPSC-derived cells compared to primary cells. However, the responses of hiPSC-derived cells were greater when cultured in advanced inflammatory conditions consisting of atheroprone hemodynamic cues with additional Tumor Necrosis Factor- α (TNF α) and oxidized low-density lipoprotein (LDL).

Protocols to differentiate pericytes in 2D cultures have been developed [13,31,36,37]. However, it has proven very difficult to establish whether these cells do in fact recapitulate true pericyte-like properties, or are simply synthetic VSMCs. Recent work by two independent groups demonstrated that pericytes can be distinguished from non-pericytes by a combination of surface markers, such as high expression of NG2, CD146, CD73 and CD44 [31,36], or alternatively

sorting of CD146^{high}CD73^{high} cells. RNA-sequencing analysis of hiPSC-derived pericytes further confirmed close similarity with primary placenta, retina and brain pericytes [31] and adipose tissue-derived pericytes [36].

2.2.3. Patient hiPSC-Derived Vascular Cells

Recently, patient-specific hiPSC-derived ECs and VSMCs were shown to be invaluable for human disease modeling applications. hiPSCs have been derived from patients suffering from fibrodysplasia ossificans progressiva (FOP) [38,39], aortic valve calcification, pulmonary arterial hypertension [40] and Marfan syndrome [41]. In addition to known mutations, disease-causing genetic variants were recently examined using this technology [42], which allowed identification of novel biological mechanisms. Combined with genome editing techniques, such as CRISPR/Cas9, mutations of interest can be corrected providing unique opportunities to study human diseases using genetically matched control lines (isogenic cell lines; Figure 1). Accurate comparison using isogenic lines can further expand our knowledge on genotype/phenotype association for more precise and personalized drug discovery. Moreover, better understanding of the disease-causing mechanisms allows for the discovery of new approaches for treatment or prevention.

2.3. Organ-on-Chip Technology for Vascular Biology

Over the past decade, many micro-engineered cell culture devices, or 'microfluidic chips', have been developed that allow *in vitro* studies of human physiology of various organ and tissue types. Typically, the microfluidic chips consist of sub-millimeter rectangular culture chambers which are connected to each other via semi-permeable barriers or micro-channels. The culture chambers either contain culture medium or 3D hydrogel, and cells are cultured on the walls of the chamber, on hydrogel surfaces, or inside the 3D hydrogel. The culture chambers in the microfluidic chips can be actively perfused at controlled flow rates and they can sometimes be actively deformed and stretched. Finally, the chips can contain integrated sensors or can be connected to external

measurements set-ups, including microscopes, in order to monitor the cells cultured inside them.

The development of microfluidic chips that contain multiple cell types have enabled *in vitro* studies of processes that are typically associated with organ-level physiology. The ultimate goal of recreating more of the key aspects of organ-level physiology ‘on-chip’ has led to the coinage of the term ‘organs-on-chips’ (or sometimes ‘microphysiological systems’) to refer to this new class of *in vitro* models. Organ-on-chip technology has also been used to capture key physiological aspects of vascular biology in systems that allow 3D network culture, that include shear stress [27,43] from fluid flow and that allow measurements of functional vascular output in an environment that is more biologically accurate than in conventional 2D static systems. In the following sections, an overview is given of advanced *in vitro* models in which organ-on-chip technology has been applied to mimic key aspects of vascular physiology.

2.3.1. Vascular Barrier Function in Chips

ECs form barriers that control passage of cells and molecules from the blood to the surrounding tissue and *vice versa*. These barriers are tissue-type dependent; for example, nutrients are reduced to essential building blocks in the gastrointestinal tract and absorbed into the local vasculature, hormones and drugs are selectively passed at the blood-brain barrier (BBB), and pulmonary microvascular cells allow for the transport of oxygen into the blood and carbon dioxide into the alveoli.

Several organ-on-chip models in which ECs are co-cultured with other cells have been developed [44]. These models have been used to test EC barrier function by directly measuring diffusion or migration of tracers [45–48] and cells, [46,47,49–53] by measuring the transendothelial electrical resistance (TEER) [46,54–56] or by performing junction-specific staining [46,55–57]. For example, when co-cultures of ECs and pulmonary epithelial cells inside lung-on-a-chip devices (Figure 2A) were stimulated with the inflammatory cytokine TNF- α , neutrophil transmigration could be induced [46]. When human brain-derived ECs in a BBB-on-a-chip (figure 2B) were stimulated with shear stress, increased barrier function was evident in TEER measurements with integrated electrodes [55,56]. Furthermore, barrier function of brain-derived ECs was also affected by co-culture in a 3D BBB-on-a-chip with pericytes or astrocytes (Figure 2C) as

measured by microscopic tracking of a fluorescent dextran tracer molecule [45]. Finally, when ECs in microfluidic devices were exposed to a vasotoxic therapeutic or to hypoxic conditions, increases in permeability were detected by quantification of gaps in cell-cell junctions [57].

Figure 2. Overview of vascular models for barrier functionality, hemostasis, thrombosis, vasculogenesis and angiogenesis in both 2D and 3D geometries. (A) Lung-on-a-chip device with two channels lined with epithelial and endothelial cells which are separated by a stretching porous membrane [46]. (B) A BBB device with two

perpendicular channels separated by a porous membrane and platinum electrodes for TEER measurements [56]. (C) Cross-section of 3D blood-brain barrier chip lined with ECs (purple) and astrocytes in the surrounding gel (green). Scale bar, 200 μm [45]. (D) Platelet aggregation in endothelialized devices, platelets have been stained using DiOC₆. Scale bar, 100 μm [66]. (E) Computational fluid dynamics (CFD) of shear stress after actuating an integrated valve, fluorescence microscopy image of ECs (blue), platelets (red) and fibrinogen (green) [77]. (F) Healthy and stenotic microfluidic channels based on CTA data filled with red food dye. CFD data of streamlines showing recirculation. Scale bar, 200 μm [72]. (G) Angiogenesis assay in collagen scaffold, nuclei (blue) and actin filaments (red) were stained. Scale bar, 50 μm [85]. (H) Vasculogenesis and angiogenesis assay for co-cultures of ECs and fibroblasts [47]. (I) Bright field images of 3D printed constructs. Fluorescence microscopy images of channels lined with various cell types: fibroblasts (blue, green), ECs (red). Scale bars: 500 μm (top), 1 mm (bottom) [92]. Reprinted and adapted with permission from: Huh et al. [46], Van der Helm et al. [56], Herland et al. [45], Westein et al. [66], Sakurai et al. [77], Costa et al. [72], Kim et al. [85], Kim et al. [47] and Kolesky et al. [92].

2.3.2. Regulation of Blood Flow, Hemostasis and Thrombosis

Microfluidic assays have been crucial in recapitulating and unraveling the complex and dynamic cellular and extracellular interactions [58–63] seen in thrombosis. For example, using the platelet adhesion molecule; von Willebrand Factor (vWF)-coated spherical microbeads in a high shear rate microfluidic environment, the role of shear rate in discoid platelet aggregation was elucidated [64]. Perfusion of platelet-free plasma through microfluidic channels with severe stenotic geometries showed the unfolding and deposition of vWF onto collagen due to an increase in shear rate [65]. Addition of ECs to these microfluidic channels with stenotic geometries demonstrated that platelet aggregation occurs after the apex of the stenosis as a result of endothelial vWF release (figure 2D) [50,66]. In addition, the role of vessel wall inflammation on platelet aggregation has been studied by direct stimulation of ECs in microfluidic channels with TNF- α [67], and even tissue-level interactions were demonstrated in an alveolus-on-a-chip system, where an endotoxin only instigated thrombosis when applied to a co-culture of epithelium and endothelium [68].

A limitation of the simplest microfluidic models of thrombosis is that they have been established in channels with rectangular cross-sections that have non-

physiological edge-effects [58]. Therefore, multiple groups have tried to better recapitulate the pathology by designing and fabricating round channel geometries [69–73]. For example, circular lumens in ECM lined with ECs and perfused with whole blood could be created by soft lithography [69] and 3D bioprinting [71], while polymethyl methacrylate (PMMA) optical fiber-molds for polydimethylsiloxane soft lithography have been used to create microchannels with circular geometries resembling aneurysms, stenoses or bifurcations [70]. Microchannels with truly defined 3D geometries based on computed tomography angiography (CTA) data have also been fabricated by 3D printing and used in thrombosis studies (Figure 2F) [72].

Hemostasis is the arrest of blood flow, and a number of studies have applied microfluidic assays to study its mechanism. For example, an extracorporeal device, with collagen-coated microfluidic channels that mimic the shear rates in occluded arterioles, was connected to an anaesthetized pig. Here, clotting times in the presence and absence of anti-platelet therapeutics were determined [74]. Hemostasis in response to vascular injury could be mimicked in microfluidic devices by using a perpendicular channel that is either coated with tissue factor and collagen or filled with a collagen scaffold [75,76]. A recent study demonstrated a microfluidic model of vascular injury by actuating a pneumatic valve in a chip with ECs and flowing blood (figure 2E) [77].

2.3.3. Vasculogenesis, Angiogenesis and Vessel Morphology

Vasculogenesis and angiogenesis refer to the formation of blood vessel networks *de novo* and by vessel sprouting from established vasculature, respectively. Both processes involve controlled breakdown of ECM and are directed by a large array of growth factors. Microfluidics allows control over patterned ECM geometries and growth factor gradients [78,79].

A typical approach to study vessel formation in microfluidic devices is to pattern 3D matrices such as collagen I, fibrin, Matrigel and alginate in specific areas of the chip [53,80–86]. Most of these assays use rows of pillars to pin the matrix in a region between two channels and allow ECs and perivascular cells to form a microvascular network (Figure 2G) either by mixing the cells with the matrix or by promoting angiogenic sprouting from one of the two channels [47].

In such devices, the role of growth factors and interstitial flow in microvascular network formation can be studied systematically [48]. Moreover, when establishing co-cultures of ECs with other cells (figure 2H), tissue-level interactions like tumor-induced angiogenesis and tumor cell extravasation can be studied [51–53,87].

Due to the limitation of artificial 2D structures like pillars and flat microchannels in these devices, there has been a focus on chips with fully engineered 3D patterns. For example, complex growth factor gradients in meandering geometries [88], 3D lumens fabricated by injection molding, viscous fingering, microneedles, 3D-printed sacrificial carbohydrate-glass and 3D-printed ECM (figure 2I) [69,89–92]. The 3D lumens can be lined with ECs and surrounded by cell-laden ECM. Studying ECs in this 3D system allows the underlying physiology to be further elucidated, for example by highlighting a new role for established vascular mechanisms such as NOTCH signalling in ECs [93,94].

2.4. Integration of iPSC-Derived Vascular Cells in Organ-on-Chip Systems

The first *in vitro* models based on hiPSC-derived vascular tissue cultured in microfluidic chips are now being reported, along with studies on primary vascular cells. Notably, these systems demonstrate that hiPSC-derived vascular cells can form lumenized vessel structures which can be perfused by fluid. In one study, human iPSC-derived ECs cultured in a custom hydrogel inside a microfluidic device generated a 3D capillary network that was stable for at least 14 days [95]. In another study, primary human ECs were mixed with human embryonic stem cell-derived pericytes inside microfluidic channels, resulting in self-organized 3D structures that displayed close interaction between pericytes and the endothelium. Moreover, the morphology of the structures could be altered by interfering with TGF β signaling and this condition can be used to mimic disease conditions, such as for example seen in the genetic disorder hereditary hemorrhagic telangiectasia (HHT) in which patients have vascular malformations [96]. Hutchinson-Gilford progeria syndrome (HGPS) is associated with premature aging that affects all cells in the body, including the vascular system due to increases in mechanical stress upon continued pulsatile blood

flow. In a study by Truskey and colleagues [97], 3D vascular constructs were generated with the use of HGPS patient derived-VMSCs. These constructs provide the potential to study the disease pathophysiology and overcome the current limitations faced from the use of murine and 2D culture. Further studies using a microfluidic organ-on-a-chip based approach was also used to re-create an *in vitro* model of HGPS using hiPSC-derived VSMCs [98]. Interestingly, hiPSC-derived VSMCs from HGPS patients cultured in microfluidic channels with continuous pulsatile stretch and relaxation stimuli showed increased DNA damage responses, senescence markers and inflammatory cytokines. Furthermore, this aging phenotype was reversed upon administration of statin (lovastatin); a drug that has been currently exploited in combination with other statins as therapeutics for life extension of HGPS patients.

Testing drugs for potential embryonic toxicity is another promising application of hiPSC-derived vascular cells in microfluidic systems [24]. In this regard, the embryonic-like phenotype of hiPSC-ECs is advantageous due to increased sensitivity to toxic compounds when compared to primary (adult) ECs. hiPSC-ECs cultured in customized tubes made of polydimethylsiloxane (PDMS) showed increased inflammatory responses upon exposure to the known cytotoxic agent (7-cyclo), that has been previously found to exhibit anti-angiogenic properties in *Xenopus* and zebrafish models [99,100]. Mechanistically, hiPSC-ECs were found to exhibit increased sensitivity due to higher VEGF Receptor 2 (VEGFR2) expression levels which is one of the targets of 7-cyclo in embryonic ECs.

2.5. Future Perspectives

2.5.1. Characterization and Quality Control of hiPSC-Derived Vascular Cells

Although hiPSC-derived vascular cells are now increasingly used as *in vitro* models of human blood vessels, there are still many challenges to their wide implementation in drug discovery in disease. For instance, despite the emergence of the variety of robust differentiation protocols there has been little cross-comparison of the derivative cells and protocols across independent labs. Consensus on standardized quality control assays and phenotypic profile would be of value. Interaction with other cells also needs further investigation; for

example, hematopoietic cells can increase angiogenesis and they may even express some EC markers so be mistaken for true ECs unless tested for their ability to form hollow (lumenized) vascular structures when embedded in hydrogels in a microfluidic system. More stringent functional assessment of hiPSC-EC identity has been recently proposed by Yoder and colleagues and outlined in their recent review [11]. There is some consensus on differentiating hiPSC-ECs but there is a greater difficulty in the differentiation and definition of VSMC and pericytes because of the lack of specific cell markers or functional assays to confirm phenotypes: expression of surface or intracellular markers is not always correlated with cell identity. Pericytes and VSMCs are recruited to newly formed EC tubes and the vascular cells mature further, which largely defines their phenotype and functionality. For these reasons, it would be interesting to consider other ways of assessing vascular cell functionality, for example by introducing standardized assays for measuring cell contractility and intracellular calcium responses (for VSMCs), as well as *in vitro* and *in vivo* vascular tube stabilization and contraction assays. Open resources on cross-comparison of published transcriptome datasets would also certainly benefit the field, and some initiatives have been established, such as the bioinformatics resource on stem cells (www.stemformatics.com).

The question also remains whether hiPSC-derived vascular cells can actually acquire mature characteristics in engineered microfluidic devices similar to those of primary cells. This is a widely recognized problem for all hiPSC derivative [101]. Unanimity on defined and standardized markers and functional assays to confirm EC and pericyte/VSMCs lineage specificity is necessary to provide cell lines that are validated as being acceptable mimics for patient-specific therapeutic research.

2.5.2. Standardization and Robustness of Organ-on-Chip Models

Most of the microfluidic *in vitro* models described in this review are currently only available to researchers and collaborators of groups that developed them. Fortunately, commercial activity in the field of organs-on-chips is changing this, with various basic systems now being commercially available [102]. Commercialization of organ-on-chip technology manufacture is beginning to

take shape but those developing this technology should take into account that future adoption by end-users will depend strongly on proper standardization. For example, designing systems with inlets, outlets and chip lay-outs that are compatible with multi-pipettes, pipetting robots and automated imaging systems, and avoiding manual fabrication of devices using PDMS-based soft lithography. PDMS is well known for its selective adsorption of drugs and medicinal entities [103]. Focus should be on large-scale fabrication and manufacturing processes based on materials like cyclic olefin copolymer (COC), polystyrene and glass [104,105]. Moreover, technology developers need to demonstrate robustness and unique added value to end-users such as. showing dose-response data for well-known experimental stimuli and by comparing this data to measurements obtained with other *in vitro* techniques and human *in vivo* data. Such comparisons can only be achieved by developing objective, quantitative endpoints, as recently carried out with measurements of TEER that allow comparison between microfluidic chips with other *in vitro* assays [106], and with measurements of pro-inflammatory cytokines in microfluidic BBB chips [45] that allow future comparison between biomarker candidates in chips and clinical samples.

2.5.3. Advanced *in vitro* Patient-Specific Disease Modeling

Disease modelling using primary cells derived from patients provides essential insight into the underlying pathology. Importantly, this provides a great advantage over the use of animal models which often do not fully encapsulate human physiology and response to stimuli. Unfortunately, the acquisition of patient tissue is difficult and sometimes impossible and moreover, the primary cells derived from the patient tissue are difficult to maintain long-term in culture. Therefore, a major advantage in using hiPSC-derived vascular cells is that they can serve as a robust source of patient-specific cells. However, genetic and ethnic background may impact responses of hiPSC-derived vascular cells to drugs and disease. In fact, even apparently “healthy controls” may have certain genetic predisposition towards some diseases that manifests only under some lifestyle conditions or later in life. Furthermore, donor age and reprogramming followed by *in vitro* expansion can introduce genomic changes [107]. Therefore, careful choice of “healthy” control lines requires extensive analysis of genomic integrity,

single nuclear polymorphism (SNP) arrays or whole genome sequencing. Genetically corrected isogenic controls are becoming a standard in modeling of monogenic diseases. Polygenic diseases are still difficult to model with hiPSCs, as this requires larger cohorts of hiPSCs from affected and unaffected individuals. However, despite being challenging, progress had been made in unexpected fields, such as modeling of psychiatric disorders with hiPSC-derived neuronal cells [108–110]. Similar studies on genetically complex cardiovascular diseases are expected using comparable approaches and selective patient inclusion. Besides genetic background, environmental factors, age and lifestyle impact disease phenotypes and drug responses. Reprogramming itself results in reversing the “biological clocks” of the cells [111–113] but may be associated with retention of “epigenetic memory” of the tissue of origin [114,115]. For late-onset diseases approaches to re-induce an aged phenotype in the cells of interest are being investigated; these include overexpression of progerin (a mutation that causes premature aging in humans) or pharmacological inhibition of telomerase [112,113].

Furthermore, vascular bed specificity of cardiovascular diseases, such as atherosclerosis, deep vein thrombosis, vascular dementia, or organ-specific failure-associated vascular dysfunction requires careful assessment of developmental origin of the cells of interest and recapitulation of tissue-specific characteristics of the differentiated cells. For instance, ECs in the vasculature are exposed to a variable fluid shear stress depending on the arterial, venous or capillary location. Tissue microenvironment is another essential driver in EC maturation during embryogenesis. Therefore, it is essential to develop culture systems that mimic as closely as possible the *in vivo* microenvironment.

2.5.4. Advanced *in vitro* Models in Drug Development

Patient-specific vascular *in vitro* models may have a significant impact on drug development, with potential applications in various phases of the drug development process: (1) disease modeling and target discovery, (2) high-throughput compound screening, (3) lead optimization and preclinical studies of pharmacokinetic properties and toxicity and (4) patient-specific studies for stratification of the population in terms of efficacy or toxicity [116]. Application of the assays in these phases of drug development enhance the chances of new

drugs being effective in trial in humans. At the same time, it places different demands on future development of the biology and technology, with some phases mainly requiring assay automation and scaling, with others mainly requiring clear disease phenotypes. In order to navigate these application-driven requirements, engineers and cell biologists will ideally actively interact with the key stakeholders in drug development, from biomedical scientists and hi-tech companies to pharmaceutical industry and regulatory agencies [117,118]. The active involvement of stakeholders and end-users, even in early technology development, will be instrumental in maximizing the impact of advanced *in vitro* models of vascular biology in drug development in the coming years.

2.5.5. Outlook

The development of advanced *in vitro* models for vascular biology based on organ-on-chip technology and hiPSC-derived cells may be recent, but the first results are very promising.

Many vascular diseases develop as a result of multiple cell type dysfunction, genetic or environmental factors. Organs-on-chips will have to become increasingly complex to capture these intricate and multifactorial aspects of vascular pathophysiology. Challenging as this may be, there is a strong sentiment in the field that by step-wise, controlled engineering of disease-related stimuli, organs-on-chips will exhibit disease phenotypes that are ever more realistic [119–121]. Eventually, the systematic inclusion of more and more disease-related factors and tissues will result in *in vitro* models that consist of multiple connected organs-on-chips that will be able to capture multi-organ or systemic aspects of vascular pathophysiology [122,123]. The advancements in the generation of patient specific iPSC types, combined with patient data in biobanks and genetic studies, as well as microfluidic technology can provide a platform for the development of *in vitro* systems to truly mimic complex disease phenotypes.

The high level of variables in animal models, along with the genetic and physiological inter-species differences, brings into question the use of *in vivo* studies as a translational tool in drug development for human disease and therapy. With advanced *in vitro* technology being developed rapidly, animal models may be reduced or eventually replaced with autologous human 3D *in vitro* systems in early drug development.

With standardized quality control in both the development of differentiation and maturation protocols for hiPSC-derived vascular cells and in the development of microfluidic technology, *in vitro* disease modelling for the understanding of disease origin, processes, biomarkers and drug responses is becoming achievable in order to provide patient-relevant data.

Figure 3. Schematic summarizing iPSC based Vessel-on-Chip technologies.

(1) It is essential to start with robust cell source with defined and standardised differentiation protocols and quality control assays to confirm cell phenotypes (2) advanced microfluidic model possessing the required functional tests where cells can be cultured to generate 3D vasculature (3) robust platform for functional tests such as drug screening, disease modelling.

2.6. References

1. Libby, P.; Theroux, P. Pathophysiology of coronary artery disease. *Circulation* 2005, *111*, 3481–3488.
2. Chalouhi, N.; Ali, M.S.; Jabbour, P.M.; Tjoumakaris, S.I.; Gonzalez, L.F.; Rosenwasser, R.H.; Koch, W.J.; Dumont, A.S. Biology of intracranial

- aneurysms: Role of inflammation. *J. Cereb. Blood Flow Metab.* 2012, 32, 1659–1676.
3. Paneni, F.; Beckman, J.A.; Creager, M.A.; Cosentino, F. Diabetes and vascular disease: Pathophysiology, clinical consequences, and medical therapy: Part i. *Eur. Heart J.* 2013, 34, 2436–2446.
4. Fukumura, D.; Jain, R.K. Tumor microvasculature and microenvironment: Targets for anti-angiogenesis and normalization. *Microvasc. Res.* 2007, 74, 72–84.
5. Benam, K.H.; Dauth, S.; Hassell, B.; Herland, A.; Jain, A.; Jang, K.-J.; Karalis, K.; Kim, H.J.; MacQueen, L.; Mahmoodian, R.; et al. Engineered In Vitro Disease Models. *Annu. Rev. Pathol. Mech. Dis.* **2015**, 10, 195–262.
6. Takahashi, K.; Tanabe, K.; Ohnuki, M.; Narita, M.; Ichisaka, T.; Tomoda, K.; Yamanaka, S. Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. *Cell* **2007**, 107, 861–872.
7. van der Helm, M.W.; van der Meer, A.D.; Eijkel, J.C.T.; van den Berg, A.; Segerink, L.I. Microfluidic organ-on-chip technology for blood-brain barrier research. *Tissue Barriers* **2016**, 4.
8. Huh, D.; Torisawa, Y.; Hamilton, G.A.; Kim, H.J.; Ingber, D.E. Microengineered physiological biomimicry: Organs-on-Chips. *Lab Chip* **2012**, 12, 2156.
9. Rustenhoven, J.; Jansson, D.; Smyth, L.C.; Dragunow, M. Brain Pericytes As Mediators of Neuroinflammation. *Trends Pharmacol. Sci.* 2017, 38, 291–304.
10. Ogura, S.; Kurata, K.; Hattori, Y.; Takase, H.; Ishiguro-Oonuma, T.; Hwang, Y.; Ahn, S.; Park, I.; Ikeda, W.; Kusuhara, S.; et al. Sustained inflammation after pericyte depletion induces irreversible blood-retina barrier breakdown. *JCI Insight* **2017**, 2.
11. Lin, Y.; Gil, C.-H.; Yoder, M.C. Differentiation, Evaluation, and Application of Human Induced Pluripotent Stem Cell-Derived Endothelial Cells. *Arterioscler. Thromb. Vasc. Biol.* **2017**, ATVBAHA.117.309962.
12. Sinha, S.; Iyer, D.; Granata, A. Embryonic origins of human vascular smooth muscle cells: implications for in vitro modeling and clinical application. *Cell. Mol. Life Sci.* **2014**, 71, 2271–88.
13. Orlova, V. V.; Drabsch, Y.; Freund, C.; Petrus-Reurer, S.; Van Den Hil, F.E.; Muenthaisong, S.; Ten Dijke, P.; Mummery, C.L. Functionality of endothelial cells and pericytes from human pluripotent stem cells demonstrated in cultured vascular plexus and zebrafish xenografts. *Arterioscler. Thromb. Vasc. Biol.* **2014**, 34, 177–186.
14. Patsch, C.; Challet-Meylan, L.; Thoma, E.C.; Urich, E.; Heckel, T.; O’Sullivan, J.F.; Grainger, S.J.; Kapp, F.G.; Sun, L.; Christensen, K.; et al. Generation of vascular endothelial and smooth muscle cells from human pluripotent stem cells. *Nat. Cell Biol.* **2015**, 17, 994–1003.
15. Sahara, M.; Hansson, E.M.; Wernet, O.; Lui, K.O.; Später, D.; Chien, K.R.

- Manipulation of a VEGF-Notch signaling circuit drives formation of functional vascular endothelial progenitors from human pluripotent stem cells. *Cell Res.* **2014**, 24, 820–841.
16. Elcheva, I.; Brok-Volchanskaya, V.; Kumar, A.; Liu, P.; Lee, J.H.; Tong, L.; Vodyanik, M.; Swanson, S.; Stewart, R.; Kyba, M.; et al. Direct induction of haematoendothelial programs in human pluripotent stem cells by transcriptional regulators. *Nat. Commun.* **2014**, 5.
 17. Palpant, N.J.; Pabon, L.; Roberts, M.; Hadland, B.; Jones, D.; Jones, C.; Moon, R.T.; Ruzzo, W.L.; Bernstein, I.; Zheng, Y.; et al. Inhibition of β -catenin signaling respecifies anterior-like endothelium into beating human cardiomyocytes. *Development* **2015**, 142, 3198–3209.
 18. Reichman, D.E.; Park, L.; Man, L.; Redmond, D.; Chao, K.; Harvey, R.P.; Taketo, M.M.; Rosenwaks, Z.; James, D. Wnt inhibition promotes vascular specification of embryonic cardiac progenitors. *Development* **2018**, 145, dev159905.
 19. Giacomelli, E.; Bellin, M.; Orlova, V. V.; Mummery, C.L. Co-Differentiation of Human Pluripotent Stem Cells-Derived Cardiomyocytes and Endothelial Cells from Cardiac Mesoderm Provides a Three-Dimensional Model of Cardiac Microtissue. In *Current Protocols in Human Genetics*; 2017; pp. 21.9.1–21.9.22 ISBN 9780471142904.
 20. Zhang, J.; Chu, L.-F.; Hou, Z.; Schwartz, M.P.; Hacker, T.; Vickerman, V.; Swanson, S.; Leng, N.; Nguyen, B.K.; Elwell, A.; et al. Functional characterization of human pluripotent stem cell-derived arterial endothelial cells. *Proc. Natl. Acad. Sci.* **2017**, 201702295.
 21. Halaidych, O. V.; Freund, C.; van den Hil, F.; Salvatori, D.C.F.; Riminucci, M.; Mummery, C.L.; Orlova, V. V. Inflammatory Responses and Barrier Function of Endothelial Cells Derived from Human Induced Pluripotent Stem Cells. *Stem Cell Reports* **2018**, 10, 1642–1656.
 22. Cochrane, A.; Kelaini, S.; Tsifaki, M.; Bojdo, J.; Vilà-González, M.; Drehmer, D.; Caines, R.; Magee, C.; Eleftheriadou, M.; Hu, Y.; et al. Quaking Is a Key Regulator of Endothelial Cell Differentiation, Neovascularization, and Angiogenesis. *Stem Cells* **2017**, 35, 952–966.
 23. Sivarapatna, A.; Ghaedi, M.; Le, A. V.; Mendez, J.J.; Qyang, Y.; Niklason, L.E. Arterial specification of endothelial cells derived from human induced pluripotent stem cells in a biomimetic flow bioreactor. *Biomaterials* **2015**, 53, 621–633.
 24. Vazão, H.; Rosa, S.; Barata, T.; Costa, R.; Pitrez, P.R.; Honório, I.; de Vries, M.R.; Papatsenko, D.; Bedito, R.; Saris, D.; et al. High-throughput identification of small molecules that affect human embryonic vascular development. *Proc. Natl. Acad. Sci.* **2017**, 114, E3022–E3031.
 25. Sriram, G.; Tan, J.Y.; Islam, I.; Rufaihah, A.J.; Cao, T. Efficient differentiation of human embryonic stem cells to arterial and venous endothelial cells under

- feeder- and serum-free conditions. *Stem Cell Res. Ther.* **2015**, 6.
26. Ando, J.; Yamamoto, K. Vascular Mechanobiology - Endothelial Cell Responses to Fluid Shear Stress. *Circ. J.* **2009**, 73, 1983–1992.
27. Sinha, R.; Le Gac, S.; Verdonchot, N.; Van Den Berg, A.; Koopman, B.; Rouwkema, J. Endothelial cell alignment as a result of anisotropic strain and flow induced shear stress combinations. *Sci. Rep.* **2016**, 6, 1–12.
28. Ohtani-Kaneko, R.; Sato, K.; Tsutiya, A.; Nakagawa, Y.; Hashizume, K.; Tazawa, H. Characterisation of human induced pluripotent stem cell-derived endothelial cells under shear stress using an easy-to-use microfluidic cell culture system. *Biomed. Microdevices* **2017**, 19.
29. Cheung, C.; Bernardo, A.S.; Trotter, M.W.B.; Pedersen, R.A.; Sinha, S. Generation of human vascular smooth muscle subtypes provides insight into embryological origin-dependent disease susceptibility. *Nat. Biotechnol.* **2012**, 30, 165–173.
30. Cheung, C.; Goh, Y.T.; Zhang, J.; Wu, C.; Guccione, E. Modeling cerebrovascular pathophysiology in amyloid-beta metabolism using neural-crest-derived smooth muscle cells. *Cell Rep.* **2014**, 9, 391–401.
31. Kumar, A.; D'Souza, S.S.; Moskvina, O. V.; Toh, H.; Wang, B.; Zhang, J.; Swanson, S.; Guo, L.W.; Thomson, J.A.; Slukvin, I.I. Specification and Diversification of Pericytes and Smooth Muscle Cells from Mesenchymoangioblasts. *Cell Rep.* **2017**, 19, 1902–1916.
32. Wanjare, M.; Kuo, F.; Gerecht, S. Derivation and maturation of synthetic and contractile vascular smooth muscle cells from human pluripotent stem cells. *Cardiovasc. Res.* **2013**, 97, 321–330.
33. Wanjare, M.; Agarwal, N.; Gerecht, S. Biomechanical strain induces elastin and collagen production in human pluripotent stem cell-derived vascular smooth muscle cells. *Am. J. Physiol. - Cell Physiol.* **2015**, 309, C271–C281.
34. Eoh, J.H.; Shen, N.; Burke, J.A.; Hinderer, S.; Xia, Z.; Schenke-Layland, K.; Gerecht, S. Enhanced elastin synthesis and maturation in human vascular smooth muscle tissue derived from induced-pluripotent stem cells. *Acta Biomater.* **2017**, 52, 49–59.
35. Collado, M.S.; Cole, B.K.; Figler, R.A.; Lawson, M.; Manka, D.; Simmers, M.B.; Hoang, S.; Serrano, F.; Blackman, B.R.; Sinha, S.; et al. Exposure of induced pluripotent stem cell-derived vascular endothelial and smooth muscle cells in coculture to hemodynamics induces primary vascular cell-like phenotypes. *Stem Cells Transl. Med.* **2017**, 6, 1673–1683.
36. Chin, C.J.; Li, S.; Corselli, M.; Casero, D.; Zhu, Y.; He, C. Bin; Hardy, R.; Péault, B.; Crooks, G.M. Transcriptionally and Functionally Distinct Mesenchymal Subpopulations Are Generated from Human Pluripotent Stem Cells. *Stem Cell Reports* **2018**.
37. Dar, A.; Domev, H.; Ben-Yosef, O.; Tzukerman, M.; Zeevi-Levin, N.; Novak, A.; Germanguz, I.; Amit, M.; Itskovitz-Eldor, J. Multipotent vasculogenic

- pericytes from human pluripotent stem cells promote recovery of murine ischemic limb. *Circulation* **2012**, 125, 87–99.
38. Cai, J.; Orlova, V. V.; Cai, X.; Eekhoff, E.M.W.; Zhang, K.; Pei, D.; Pan, G.; Mummery, C.L.; Ten Dijke, P. Induced Pluripotent Stem Cells to Model Human Fibrodysplasia Ossificans Progressiva. *Stem Cell Reports* **2015**, 5, 963–970.
 39. Hino, K.; Ikeya, M.; Horigome, K.; Matsumoto, Y.; Ebise, H.; Nishio, M.; Sekiguchi, K.; Shibata, M.; Nagata, S.; Matsuda, S.; et al. Neofunction of ACVR1 in fibrodysplasia ossificans progressiva. *Proc. Natl. Acad. Sci.* **2015**, 112, 15438–15443.
 40. Gu, M.; Shao, N.Y.; Sa, S.; Li, D.; Termglinchan, V.; Ameen, M.; Karakikes, I.; Sosa, G.; Grubert, F.; Lee, J.; et al. Patient-Specific iPSC-Derived Endothelial Cells Uncover Pathways that Protect against Pulmonary Hypertension in BMPR2 Mutation Carriers. *Cell Stem Cell* **2017**, 20, 490-504.e5.
 41. Granata, A.; Serrano, F.; Bernard, W.G.; McNamara, M.; Low, L.; Sastry, P.; Sinha, S. An iPSC-derived vascular model of Marfan syndrome identifies key mediators of smooth muscle cell death. *Nat. Genet.* **2017**, 49, 97–109.
 42. Gupta, R.M.; Hadaya, J.; Trehan, A.; Zekavat, S.M.; Roselli, C.; Klarin, D.; Emdin, C.A.; Hilvering, C.R.E.; Bianchi, V.; Mueller, C.; et al. A Genetic Variant Associated with Five Vascular Diseases Is a Distal Regulator of Endothelin-1 Gene Expression. *Cell* **2017**, 170, 522-533.e15.
 43. Zohar, B.; Blinder, Y.; Mooney, D.J.; Levenberg, S. Flow-Induced Vascular Network Formation and Maturation in Three-Dimensional Engineered Tissue. *ACS Biomater. Sci. Eng.* **2017**, acsbiomaterials.7b00025.
 44. Kim, S.; Kim, W.; Lim, S.; Jeon, J. Vasculature-On-A-Chip for In Vitro Disease Models. *Bioengineering* **2017**, 4, 8.
 45. Herland, A.; Van Der Meer, A.D.; FitzGerald, E.A.; Park, T.E.; Sleeboom, J.J.F.; Ingber, D.E. Distinct contributions of astrocytes and pericytes to neuroinflammation identified in a 3D human blood-brain barrier on a chip. *PLoS One* **2016**, 11, 1–21.
 46. Huh D, Matthews B, Mammoto A, Montoya-Zavala M, Hsin H, I.D. Reconstituting Organ-Level Lung. *Science* (80-.). **2010**, 328, 1662–1668.
 47. Kim, S.; Lee, H.; Chung, M.; Jeon, N.L. Engineering of functional, perfusable 3D microvascular networks on a chip. *Lab Chip* **2013**, 13, 1489.
 48. Kim, S.; Chung, M.; Ahn, J.; Lee, S.; Jeon, N.L. Interstitial flow regulates the angiogenic response and phenotype of endothelial cells in a 3D culture model. *Lab Chip* **2016**, 16, 4189–4199.
 49. Cho, H.; Seo, J.H.; Wong, K.H.K.; Terasaki, Y.; Park, J.; Bong, K.; Arai, K.; Lo, E.H.; Irimia, D. Three-Dimensional Blood-Brain Barrier Model for in vitro Studies of Neurovascular Pathology. *Sci. Rep.* **2015**, 5, 15222.
 50. Menon, N.V.; Tay, H.M.; Wee, S.N.; Li, K.H.H.; Hou, H.W. Micro-engineered perfusable 3D vasculatures for cardiovascular diseases. *Lab Chip* **2017**, 17,

- 2960–2968.
51. Jeon, J.S.; Bersini, S.; Gilardi, M.; Dubini, G.; Charest, J.L.; Moretti, M.; Kamm, R.D.; Jeon, J.S.; Bersini, S.; Gilardi, M.; et al. Correction for Jeon et al., Human 3D vascularized organotypic microfluidic assays to study breast cancer cell extravasation. *Proc. Natl. Acad. Sci.* **2015**, *112*, E818–E818.
52. Zervantonakis, I.K.; Hughes-Alford, S.K.; Charest, J.L.; Condeelis, J.S.; Gertler, F.B.; Kamm, R.D. Three-dimensional microfluidic model for tumor cell intravasation and endothelial barrier function. *Proc. Natl. Acad. Sci.* **2012**, *109*, 13515–13520.
53. Chung, S.; Sudo, R.; Mack, P.J.; Wan, C.-R.; Vickerman, V.; Kamm, R.D. Cell migration into scaffolds under co-culture conditions in a microfluidic platform. *Lab Chip* **2009**, *9*, 269–275.
54. Kim, H.J.; Li, H.; Collins, J.J.; Ingber, D.E. Contributions of microbiome and mechanical deformation to intestinal bacterial overgrowth and inflammation in a human gut-on-a-chip. *Proc. Natl. Acad. Sci.* **2016**, *113*, E7–E15.
55. Griep, L.M.; Wolbers, F.; De Wagenaar, B.; Ter Braak, P.M.; Weksler, B.B.; Romero, I.A.; Couraud, P.O.; Vermes, I.; Van Der Meer, A.D.; Van Den Berg, A. BBB on CHIP: Microfluidic platform to mechanically and biochemically modulate blood-brain barrier function. *Biomed. Microdevices* **2013**, *15*, 145–150.
56. van der Helm, M.W.; Odijk, M.; Frimat, J.P.; van der Meer, A.D.; Eijkel, J.C.T.; van den Berg, A.; Segerink, L.I. Direct quantification of transendothelial electrical resistance in organs-on-chips. *Biosens. Bioelectron.* **2016**, *85*, 924–929.
57. Abaci, H.E.; Shen, Y.I.; Tan, S.; Gerecht, S. Recapitulating physiological and pathological shear stress and oxygen to model vasculature in health and disease. *Sci. Rep.* **2014**, *4*, 1–9.
58. Westein, E.; De Witt, S.; Lamers, M.; Cosemans, J.M.E.M.; Heemskerk, J.W.M. Monitoring in vitro thrombus formation with novel microfluidic devices. *Platelets* **2012**, *23*, 501–509.
59. Van Kruchten, R.; Cosemans, J.M.E.M.; Heemskerk, J.W.M. Measurement of whole blood thrombus formation using parallel-plate flow chambers a practical guide. *Platelets* **2012**, *23*, 229–242.
60. Coenen, D.M.; Mastenbroek, T.G.; Cosemans, J.M.E.M. Platelet interaction with activated endothelium: mechanistic insights from microfluidics. *Blood* **2017**, *130*, 2819–2828.
61. Pandian, N.K.R.; Mannino, R.G.; Lam, W.A.; Jain, A. Thrombosis-on-a-chip: Prospective impact of microphysiological models of vascular thrombosis. *Curr. Opin. Biomed. Eng.* **2018**, *5*, 29–34.
62. Zhu, S.; Herbig, B.A.; Li, R.; Colace, T. V.; Muthard, R.W.; Neeves, K.B.; Diamond, S.L. In microfluidico: Recreating in vivo hemodynamics using miniaturized devices. *Biorheology* **2015**, *52*, 303–318.
63. Mannino, R.G.; Pandian, N.K.; Jain, A.; Lam, W.A. Engineering “Endothelialized” Microfluidics for Investigating Vascular and Hematologic

- Processes Using Non-Traditional Fabrication Techniques. *Curr. Opin. Biomed. Eng.* **2017**, *5*, 13–20.
64. Nesbitt, W.S.; Westein, E.; Tovar-Lopez, F.J.; Tolouei, E.; Mitchell, A.; Fu, J.; Carberry, J.; Fouras, A.; Jackson, S.P. A shear gradient-dependent platelet aggregation mechanism drives thrombus formation. *Nat Med* **2009**, *15*, 665–673.
 65. Colace, T. V.; Diamond, S.L. Direct observation of von Willebrand factor elongation and fiber formation on collagen during acute whole blood exposure to pathological flow. *Arterioscler. Thromb. Vasc. Biol.* **2013**, *33*, 105–113.
 66. Westein, E.; van der Meer, A.D.; Kuijpers, M.J.E.; Frimat, J.-P.; van den Berg, A.; Heemskerk, J.W.M. Atherosclerotic geometries exacerbate pathological thrombus formation poststenosis in a von Willebrand factor-dependent manner. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 1357–62.
 67. Jain, A.; van der Meer, A.D.; Papa, A.L.; Barrile, R.; Lai, A.; Schlechter, B.L.; Otieno, M.A.; Loudon, C.S.; Hamilton, G.A.; Michelson, A.D.; et al. Assessment of whole blood thrombosis in a microfluidic device lined by fixed human endothelium. *Biomed. Microdevices* **2016**, *18*, 1–7.
 68. Jain, A.; Barrile, R.; van der Meer, A.D.; Mammoto, A.; Mammoto, T.; De Ceunynck, K.; Aisiku, O.; Otieno, M.A.; Loudon, C.S.; Hamilton, G.A.; et al. Primary Human Lung Alveolus-on-a-chip Model of Intravascular Thrombosis for Assessment of Therapeutics. *Clin. Pharmacol. Ther.* **2018**, *103*, 332–340.
 69. Zheng, Y.; Chen, J.; Craven, M.; Choi, N.W.; Totorica, S.; Diaz-Santana, A.; Kermani, P.; Hempstead, B.; Fischbach-Teschl, C.; López, J.A.; et al. In vitro microvessels for the study of angiogenesis and thrombosis. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 9342–7.
 70. Mannino, R.G.; Myers, D.R.; Ahn, B.; Wang, Y.; Margo Rollins; Gole, H.; Lin, A.S.; Guldberg, R.E.; Giddens, D.P.; Timmins, L.H.; et al. “Do-it-yourself in vitro vasculature that recapitulates in vivo geometries for investigating endothelial-blood cell interactions”. *Sci. Rep.* **2015**, *5*, 12401.
 71. Zhang, Y.S.; Davoudi, F.; Walch, P.; Manbachi, A.; Luo, X.; Dell’Erba, V.; Miri, A.K.; Albadawi, H.; Arneri, A.; Li, X.; et al. Bioprinted thrombosis-on-a-chip. *Lab Chip* **2016**, *16*, 4097–4105.
 72. Costa, P.F.; Albers, H.J.; Linssen, J.E.A.; Middelkamp, H.H.T.; van der Hout, L.; Passier, R.; van den Berg, A.; Malda, J.; van der Meer, A.D. Mimicking arterial thrombosis in a 3D-printed microfluidic in vitro vascular model based on computed tomography angiography data. *Lab Chip* **2017**, *17*.
 73. van Duinen, V.; Trietsch, S.J.; Joore, J.; Vulto, P.; Hankemeier, T. Microfluidic 3D cell culture: From tools to tissue models. *Curr. Opin. Biotechnol.* **2015**, *35*, 118–126.
 74. Jain, A.; Graveline, A.; Waterhouse, A.; Vernet, A.; Flaumenhaft, R.; Ingber,

- D.E. A shear gradient-activated microfluidic device for automated monitoring of whole blood haemostasis and platelet function. *Nat. Commun.* **2016**, *7*.
75. Muthard, R.W.; Diamond, S.L. Side view thrombosis microfluidic device with controllable wall shear rate and transthrombus pressure gradient. *Lab Chip* **2013**, *13*, 1883–91.
76. Schoeman, R.M.; Rana, K.; Danes, N.; Lehmann, M.; Di Paola, J.A.; Fogelson, A.L.; Leiderman, K.; Neeves, K.B. A Microfluidic Model of Hemostasis Sensitive to Platelet Function and Coagulation. *Cell. Mol. Bioeng.* **2017**, *10*, 3–15.
77. Sakurai, Y.; Hardy, E.T.; Ahn, B.; Tran, R.; Fay, M.E.; Ciciliano, J.C.; Mannino, R.G.; Myers, D.R.; Qiu, Y.; Carden, M.A.; et al. A microengineered vascularized bleeding model that integrates the principal components of hemostasis. *Nat. Commun.* **2018**, 1–9.
78. Rouwkema, J.; Khademhosseini, A. Vascularization and Angiogenesis in Tissue Engineering: Beyond Creating Static Networks. *Trends Biotechnol.* **2016**, *34*, 733–745.
79. Chung, S.; Sudo, R.; Vickerman, V.; Zervantonakis, I.K.; Kamm, R.D. Microfluidic platforms for studies of angiogenesis, cell migration, and cell-cell interactions: Sixth international bio-fluid mechanics symposium and workshop March 28-30, 2008 Pasadena, California. *Ann. Biomed. Eng.* **2010**, *38*, 1164–1177.
80. Sudo, R.; Chung, S.; Zervantonakis, I.K.; Vickerman, V.; Toshimitsu, Y.; Griffith, L.G.; Kamm, R.D. Transport-mediated angiogenesis in 3D epithelial coculture. *FASEB J.* **2009**, *23*, 2155–2164.
81. Vickerman, V.; Blundo, J.; Chung, S.; Kamm, R. Design, fabrication and implementation of a novel multi-parameter control microfluidic platform for three-dimensional cell culture and real-time imaging. *Lab Chip* **2008**, *8*, 1468.
82. Shin, Y.; Jeon, J.S.; Han, S.; Jung, G.-S.; Shin, S.; Lee, S.-H.; Sudo, R.; Kamm, R.D.; Chung, S. In vitro 3D collective sprouting angiogenesis under orchestrated ANG-1 and VEGF gradients. *Lab Chip* **2011**, *11*, 2175.
83. Jeong, G.S.; Han, S.; Shin, Y.; Kwon, G.H.; Kamm, R.D.; Lee, S.H.; Chung, S. Sprouting angiogenesis under a chemical gradient regulated by interactions with an endothelial monolayer in a microfluidic platform. *Anal. Chem.* **2011**, *83*, 8454–8459.
84. Huang, C.P.; Lu, J.; Seon, H.; Lee, A.P.; Flanagan, L.A.; Kim, H.-Y.; Putnam, A.J.; Jeon, N.L. Engineering microscale cellular niches for three-dimensional multicellular co-cultures. *Lab Chip* **2009**, *9*, 1740.
85. Kim, C.; Kasuya, J.; Jeon, J.; Chung, S.; Kamm, R.D. A quantitative microfluidic angiogenesis screen for studying anti-angiogenic therapeutic drugs. *Lab Chip* **2015**, *15*, 301–310.
86. Chan, J.M.; Zervantonakis, I.K.; Rimchala, T.; Polacheck, W.J.; Whisler, J.;

- Kamm, R.D. Engineering of In Vitro 3D Capillary Beds by Self-Directed Angiogenic Sprouting. *PLoS One* **2012**, 7, 1–11.
87. Zheng, Y.; Sun, Y.; Yu, X.; Shao, Y.; Zhang, P.; Dai, G.; Fu, J. Angiogenesis in Liquid Tumors: An In Vitro Assay for Leukemic-Cell-Induced Bone Marrow Angiogenesis. *Adv. Healthc. Mater.* **2016**, 5, 1014–1024.
88. Baker, B.M.; Trappmann, B.; Stapleton, S.C.; Toro, E.; Chen, C.S. Microfluidics embedded within extracellular matrix to define vascular architectures and pattern diffusive gradients. *Lab Chip* **2013**, 13, 3246.
89. Nguyen, D.-H.T.; Stapleton, S.C.; Yang, M.T.; Cha, S.S.; Choi, C.K.; Galie, P.A.; Chen, C.S. Biomimetic model to reconstitute angiogenic sprouting morphogenesis in vitro. *Proc. Natl. Acad. Sci.* **2013**, 110, 6712–6717.
90. Bischel, L.L.; Young, E.W.K.; Mader, B.R.; Beebe, D.J. Tubeless microfluidic angiogenesis assay with three-dimensional endothelial-lined microvessels. *Biomaterials* **2013**, 34, 1471–1477.
91. Miller, J.S.; Stevens, K.R.; Yang, M.T.; Baker, B.M.; Nguyen, D.-H.T.; Cohen, D.M.; Toro, E.; Chen, A.A.; Galie, P.A.; Yu, X.; et al. Rapid casting of patterned vascular networks for perfusable engineered three-dimensional tissues. *Nat. Mater.* **2012**, 11, 768–774.
92. Kolesky, D.B.; Truby, R.L.; Gladman, A.S.; Busbee, T.A.; Homan, K.A.; Lewis, J.A. 3D bioprinting of vascularized, heterogeneous cell-laden tissue constructs. *Adv. Mater.* **2014**, 26.
93. Polacheck, W.J.; Kutys, M.L.; Yang, J.; Eyckmans, J.; Wu, Y.; Vasavada, H.; Hirschi, K.K.; Chen, C.S. A non-canonical Notch complex regulates adherens junctions and vascular barrier function. *Nature* **2017**, 552, 258–262.
94. Mack, J.J.; Mosqueiro, T.S.; Archer, B.J.; Jones, W.M.; Sunshine, H.; Faas, G.C.; Briot, A.; Aragón, R.L.; Su, T.; Romay, M.C.; et al. NOTCH1 is a mechanosensor in adult arteries. *Nat. Commun.* **2017**, 8, 1–18.
95. Kurokawa, Y.K.; Yin, R.T.; Shang, M.R.; Shirure, V.S.; Moya, M.L.; George, S.C. Human iPSC-derived endothelial cells for 3D microphysiological systems. *Tissue Eng. Part C Methods* **2017**, ten.TEC.2017.0133.
96. Meer, A.D. van der; Orlova, V. V.; Dijke, P. ten; Berg, A. van den; Mummery, C.L. Three-dimensional co-cultures of human endothelial cells and embryonic stem cell-derived pericytes inside a microfluidic device. *Lab Chip* **2013**, 13, 3562–3568.
97. Atchison, L.; Zhang, H.; Cao, K.; Truskey, G.A. A Tissue Engineered Blood Vessel Model of Hutchinson-Gilford Progeria Syndrome Using Human iPSC-derived Smooth Muscle Cells. *Sci. Rep.* **2017**, 7, 1–12.
98. Ribas, J.; Zhang, Y.S.; Pitrez, P.R.; Leijten, J.; Miscuglio, M.; Rouwkema, J.; Dokmeci, M.R.; Nissan, X.; Ferreira, L.; Khademhosseini, A. Biomechanical Strain Exacerbates Inflammation on a Progeria-on-a-Chip Model. *Small* **2017**, 13.
99. Gallardo, V.E.; Varshney, G.K.; Lee, M.; Bupp, S.; Xu, L.; Shinn, P.; Crawford,

- N.P.; Inglese, J.; Burgess, S.M. Phenotype-driven chemical screening in zebrafish for compounds that inhibit collective cell migration identifies multiple pathways potentially involved in metastatic invasion. *Dis. Model. Mech.* **2015**, *8*, 565–576.
100. Kälén, R.E.; Bänziger-Tobler, N.E.; Detmar, M.; Brändli, A.W. An *in vivo* chemical library screen in *Xenopus* tadpoles reveals novel pathways involved in angiogenesis and lymphangiogenesis. *Blood* **2009**, *114*, 1110–1122.
 101. Veerman, C.C.; Kosmidis, G.; Mummery, C.L.; Casini, S.; Verkerk, A.O.; Bellin, M. Immaturity of Human Stem-Cell-Derived Cardiomyocytes in Culture: Fatal Flaw or Soluble Problem? *Stem Cells Dev.* **2015**, *24*, 1035–1052.
 102. Zhang, B.; Radisic, M. Organ-on-a-chip devices advance to market. *Lab Chip* **2017**, *17*, 2395–2420.
 103. van Meer, B.J.; de Vries, H.; Firth, K.S.A.; van Weerd, J.; Tertoolen, L.G.J.; Karperien, H.B.J.; Jonkheijm, P.; Denning, C.; IJzerman, A.P.; Mummery, C.L. Small molecule absorption by PDMS in the context of drug response bioassays. *Biochem. Biophys. Res. Commun.* **2017**, *482*, 323–328.
 104. Becker, H.; Gärtner, C. Polymer microfabrication technologies for microfluidic systems. *Anal. Bioanal. Chem.* **2008**, *390*, 89–111.
 105. Berthier, E.; Young, E.W.K.; Beebe, D. Engineers are from PDMS-land, Biologists are from Polystyrenia. *Lab Chip* **2012**, *12*, 1224.
 106. Odijk, M.; van der Meer, A.D.; Levner, D.; Kim, H.J.; van der Helm, M.W.; Segerink, L.I.; Frimat, J.-P.; Hamilton, G.A.; Ingber, D.E.; van den Berg, A. Measuring direct current trans-epithelial electrical resistance in organ-on-a-chip microsystems. *Lab Chip* **2015**, *15*, 745–752.
 107. Gore, A.; Li, Z.; Fung, H.-L.; Young, J.E.; Agarwal, S.; Antosiewicz-Bourget, J.; Canto, I.; Giorgetti, A.; Israel, M.A.; Kiskinis, E.; et al. Somatic coding mutations in human induced pluripotent stem cells. *Nature* **2011**, *471*, 63–67.
 108. de Vrij, F.M.; Bouwkamp, C.G.; Gunhanlar, N.; Shpak, G.; Lendemeijer, B.; Baghdadi, M.; Gopalakrishna, S.; Ghazvini, M.; Li, T.M.; Quadri, M.; et al. Candidate CSPG4 mutations and induced pluripotent stem cell modeling implicate oligodendrocyte progenitor cell dysfunction in familial schizophrenia. *Mol. Psychiatry* **2018**, 1–15.
 109. Hoffman, G.E.; Hartley, B.J.; Flaherty, E.; Ladrán, I.; Gochman, P.; Ruderfer, D.M.; Stahl, E.A.; Rapoport, J.; Sklar, P.; Brennand, K.J. Transcriptional signatures of schizophrenia in hiPSC-derived NPCs and neurons are concordant with post-mortem adult brains. *Nat. Commun.* **2017**, *8*.
 110. Wen, Z.; Nguyen, H.N.; Guo, Z.; Lalli, M.A.; Wang, X.; Su, Y.; Kim, N.S.; Yoon, K.J.; Shin, J.; Zhang, C.; et al. Synaptic dysregulation in a human iPSC cell model of mental disorders. *Nature* **2014**, *515*, 414–418.
 111. Cornacchia, D.; Studer, L. Back and forth in time: Directing age in iPSC-derived lineages. *Brain Res.* **2017**, *1656*, 14–26.
 112. Miller, J.D.; Ganat, Y.M.; Kishinevsky, S.; Bowman, R.L.; Liu, B.; Tu, E.Y.;

- Mandal, P.K.; Vera, E.; Shim, J.W.; Kriks, S.; et al. Human iPSC-based modeling of late-onset disease via progerin-induced aging. *Cell Stem Cell* **2013**, *13*, 691–705.
113. Vera, E.; Bosco, N.; Studer, L. Generating Late-Onset Human iPSC-Based Disease Models by Inducing Neuronal Age-Related Phenotypes through Telomerase Manipulation. *Cell Rep.* **2016**, *17*, 1184–1192.
114. Roost, M.S.; Sliker, R.C.; Bialecka, M.; Van Iperen, L.; Gomes Fernandes, M.M.; He, N.; Suchiman, H.E.D.; Szuhai, K.; Carlotti, F.; De Koning, E.J.P.; et al. DNA methylation and transcriptional trajectories during human development and reprogramming of isogenic pluripotent stem cells. *Nat. Commun.* **2017**, *8*.
115. Nishizawa, M.; Chonabayashi, K.; Nomura, M.; Tanaka, A.; Nakamura, M.; Inagaki, A.; Nishikawa, M.; Takei, I.; Oishi, A.; Tanabe, K.; et al. Epigenetic Variation between Human Induced Pluripotent Stem Cell Lines Is an Indicator of Differentiation Capacity. *Cell Stem Cell* **2016**, *19*, 341–354.
116. Esch, E.W.; Bahinski, A.; Huh, D. Organs-on-chips at the frontiers of drug discovery. *Nat. Rev. Drug Discov.* **2015**, *14*, 248–260.
117. Middelkamp, H.H.T.; van der Meer, A.D.; Hummel, J.M.; Stamatialis, D.F.; Mummery, C.L.; Passier, R.; IJzerman, M.J. Organs-on-Chips in Drug Development: The Importance of Involving Stakeholders in Early Health Technology Assessment. *Appl. Vitro. Toxicol.* **2016**, *2*, 74–81.
118. Bahinski, A.; Horland, R.; Huh, D.; Mummery, C.; Tagle, D.A.; MacGill, T. The Promise and Potential of “Organs-on-Chips” as Preclinical Models. *Appl. Vitro. Toxicol.* **2015**, *1*, 235–242.
119. Meer, A.D. van der; Berg, A. van den Organs-on-chips: breaking the in vitro impasse. *Integr. Biol.* **2012**, *4*, 461.
120. Ingber, D.E. Developmentally inspired human “organs on chips”. *Development* **2018**, 10–13.
121. Ingber, D.E. Reverse Engineering Human Pathophysiology with Organs-on-Chips. *Cell* **2016**, *164*, 1105–1109.
122. Huh, D.; Hamilton, G.A.; Ingber, D.E. From 3D cell culture to organs-on-chips. *Trends Cell Biol.* **2011**, *21*, 745–754.
123. Esch, M.B.; King, T.L.; Shuler, M.L. The Role of Body-on-a-Chip Devices in Drug and Toxicity Studies. *Annu. Rev. Biomed. Eng.* **2011**, *13*, 55–72.

Chapter 3

Mimicking Arterial Thrombosis in a 3D-Printed Microfluidic *in vitro* Vascular Model Based on Computed Tomography Angiography Data

Chapter based on: Costa, P. F.⁺, Albers, H. J. ⁺, Linssen, J. E. ⁺, Middelkamp, H. H.T., Van Der Hout, L., Passier, R., Van den Berg, A. , Malda, J., & Van Der Meer, A. D. (2017). Mimicking arterial thrombosis in a 3D-printed microfluidic in vitro vascular model based on computed tomography angiography data. *Lab on a Chip*, 17(16), 2785-2792. ⁺Shared first-authorship.

Abstract

Arterial thrombosis is the main instigating factor of heart attacks and strokes, which result in over 14 million deaths worldwide every year. The mechanism of thrombosis involves factors from the blood and the vessel wall, and it also relies strongly on 3D vessel geometry and local blood flow patterns. Microfluidic chip-based vascular models allow controlled *in vitro* studies of the interaction between vessel wall and blood in thrombosis, but until now, they could not fully recapitulate the 3D geometry and blood flow patterns of real-life healthy or diseased arteries. Here we present a method for fabricating microfluidic chips containing miniaturized vascular structures that closely mimic architectures found in both healthy and stenotic blood vessels. By applying stereolithography (SLA) 3D printing of computed tomography angiography (CTA) data, 3D vessel constructs were produced with diameters of 400 μm , and resolutions as low as 25 μm . The 3D-printed templates in turn were used as moulds for polydimethylsiloxane (PDMS)-based soft lithography to create microfluidic chips containing miniaturized replicates of *in vivo* vessel geometries. By applying Computational Fluid Dynamics (CFD) modeling a correlation in terms of flow fields and local wall shear rate was found between the original and miniaturized artery. The walls of the microfluidic chips were coated with human umbilical vein endothelial cells (HUVECs) which formed a confluent monolayer as confirmed by confocal fluorescence microscopy. The endothelialised microfluidic devices, with healthy and stenotic geometries, were perfused with human whole blood with fluorescently labeled platelets at physiologically relevant shear rates. After 15 minutes of perfusion the healthy geometries showed no sign of thrombosis, while the stenotic geometries did induce thrombosis at and downstream of the stenotic area. Overall, the novel methodology reported here, overcomes important design limitations found in typical 2D wafer-based soft lithography microfabrication techniques and shows great potential for controlled studies of the role of 3D vessel geometries and blood flow patterns in arterial thrombosis.

3.1. Introduction

Arterial thrombosis is the main cause for heart attacks and strokes, which together cause over 14 million deaths worldwide every year [1]. Thrombosis is a complex process, which is instigated by multiple factors both genetic and acquired: blood-borne factors, vessel wall dysfunction and fluid dynamical effects. Animal models are the most popular experimental tool to study the pathophysiology of thrombosis in laboratory settings. While animal models mimic the mechanistic multifactorial complexity found in arterial thrombosis, they do not fully recapitulate human physiology or human disease. For example, rodent platelet biology [2] and coagulation dynamics [3] display inter-species differences, and mice arteries typically undergo wall shear stresses that are an order of magnitude higher [4] than those in humans. Furthermore, thrombosis in mice can only be instigated in the largest arteries by using ferric chloride or by laser-induced injury [5–8], limiting their use in studying thrombosis in specific, well-controlled 3D vascular geometries. Therefore, engineered *in vitro* blood flow chambers, perfused with human blood, are often used to complement or confirm results from *in vivo* models [9,10].

In recent years, microfluidic organs-on-chips emerged as new engineered *in vitro* models that capture aspects of human physiology with an unprecedented level of realism [11–13]. Microfluidic technology has also been used extensively to create new *in vitro* models for studying thrombosis [14–18]. For example, when microfluidic channels with cultured endothelial cells and geometric constrictions mimicking stenotic vessels were perfused with human whole blood, platelet aggregates tend to form specifically in the outlet zone of the constriction [14,15,19]. Mimicking arterial physiology also encompasses applying an arterial shear rate to the endothelial cells and blood. It has been shown that shear rate is very important in von Willebrand Factor activation and thus influences platelet adhesion and aggregation [14,15,20,21]. However, due to inherent limitations posed by 2D wafer-based microfabrication techniques of microfluidic chips, the channels in almost all on-chip thrombosis models inevitably possess planar configurations with rectangular cross-sections instead of the typical 3D, round cross-section blood vessel structures found *in vivo* [11,22]. A number of strategies have been employed to produce chips that contain microfluidic channels with circular cross-sections. For example, acrylic optical fibres in combination with

standard soft lithography can be used to fabricate stylized geometries reminiscent of vessel stenoses and aneurysms [19]. Moreover, microfabrication techniques, such as 3D bioprinting and gelatin-based soft lithography, have also been used to engineer microfluidic channels with circular cross-sections, which were then perfused with human blood to study thrombosis and thrombolysis [23,24].

So far, none of these studies yielded microfluidic chips in which thrombosis can be studied in geometries that are truly representative of human vessel geometries. This type of 3D complexity is essential to accurately mimic the *in vivo* flow patterns which are fundamental in the mechanism of thrombosis [14,25,26].

Three-dimensional printing has the potential to revolutionize the field of microfluidics by allowing to overcome the limitations posed by conventional 2D wafer-based microfabrication techniques [22]. Furthermore, it allows the fabrication of fully 3D structures, which closely mimic the natural shape of complex human tissue structures and could, therefore, also be useful in replicating the architecture of human blood vessels [27–29]. Stereolithography (SLA) in particular could be a suitable technique to fabricate architecturally complex vessel models, since it can reach very high printing resolutions and has already been successfully used in manufacturing microfluidic chips [30–32]. In SLA-based technologies, 3D constructs are manufactured in a layer-by-layer fashion from liquid photolabile polymers (resins), which are cured at specific locations through exposure to light [32,33].

In this study, we describe an advanced method to produce microfluidic chips that replicate the exact 3D architecture of both healthy and stenotic coronary arteries. Our method relies on SLA-based fabrication of miniaturized models of a coronary artery based on computed tomography angiography (CTA) imaging data. The printed models are used as moulds in polydimethylsiloxane (PDMS)-based soft lithography to produce microfluidic chips that contain exact replicas of the arterial architecture, as well as inlets and outlets to allow perfusion of the chips. By replicating the geometries found *in vivo* it becomes possible to overcome the limitations usually encountered when using square channel geometries, *e.g.* unrealistic flow profiles and fibrin formation in low-flow zones in channel corners. Moreover, we demonstrate that microfluidic chips produced with our method are compatible with cell culture and high-resolution confocal microscopy by seeding human umbilical vein endothelial cells (HUVECs)

followed by image analysis. Finally, as a proof-of-principle, we assessed the effect of vessel geometries on platelet aggregation by perfusing healthy and stenotic vessel designs with human whole blood under real arterial shear rates.

3.2. Materials & Methods

3.2.1. Coronary Artery 3D Models

Anonymised thoracic CTA scans were acquired from a free online digital imaging and communication in medicine (DICOM) library [34]. DICOM files consist of stacks of 2D tissue image sections which, when combined, can be used to generate 3D models. The utilized DICOM files were read and segmented using 3D Slicer (<http://www.slicer.org>) [35]. In short, by applying thresholding functions, all irrelevant tissue regions were excluded in order to only highlight vascular tissue. The result from this threshold was then converted to a 3D model by using the “model making” option in 3D Slicer.

Directly after segmentation, many artefact volumes remained from surrounding tissue. The 3D model was, therefore, further processed in computer-aided design (CAD) software Blender (Blender foundation, Amsterdam, The Netherlands, version 2.72) to remove artefacts and smoothen the surface. Through specific selection of meshes within Edit Mode, the residual surrounding tissue geometries and artefacts were removed. The model was subsequently smoothened within Sculpt Mode using the ‘F smooth function’. After downsizing, this resulted in a 3D vessel model with a channel diameter of 400 μm (Figure 1A).

Stenosis models with 55% and 67% constrictions were then further created by narrowing a straight segment of the channel to diameters of 180 μm and 140 μm respectively (Figure 1B, 55% not shown). This step was performed in Blender with the Sculpt Mode by selecting the ‘F-inflate/deflate’ and ‘F-smooth’ functions.

3.2.2. Microfluidic Device Design and Fabrication

Microfluidic devices were fabricated by common PDMS-based soft lithography using moulds created by SLA [22,31].

Figure 1. Three-dimensional models of a healthy and stenotic vessel and chip fabrication. Three-dimensional render of a healthy (A) and stenosis (B) model as acquired from segmentation and after manual editing in 3D modelling software. Insets show high magnifications of a straight section and a stenotic section, respectively. (C) Top: Image of the 3D model, in grey is depicted the outer walls of the mould, in blue the print support structures and in red the microfluidic channel. Bottom: Photograph of the printed model. Width of the entire model is 1.2 cm. (D) Close-up pictures of a healthy and stenotic print, 370 μ m in diameter (healthy) and 120 μ m (stenosis). (E) Cast PDMS chip after mould removal, channel diameter 370 μ m. (F) close-up of a stenotic and healthy channel after PDMS casting. The red colouring is made with food dye. Scale bars coincide with 370 μ m.

The full model for 3D printing contained the 3D vessel models, as well as inlets, outlets and a box-like container and was designed in Blender CAD software. The generated 3D vessel models were further extended with cylindrical sections so they would fit the length (15 mm) of the box-like container. The container (width 10 mm) was composed of six 4 mm thick walls which were designed to restrict the PDMS into a confined volume upon casting and moulding in later steps. Furthermore, the full 3D model contained inlets and outlets (diameter 1 mm, height 5 mm), and support structures (width $300 \times 500 \mu\text{m}$, height 1.8 mm) connected to the inlets, outlets and vessel models to allow accurate printing of the suspended section of the 3D artery model. The maximum amount of overhang in an SLA model is roughly 45° , and any structures positioned below this angle regularly fail to print. The final curvature of the printed model can exceed 45° as long as each subsequent layer does not exceed the 45° overhang compared to the previous layer. The inlets and outlets were positioned at a horizontal angle (*i.e.* $0/180^\circ$) and therefore support structures were needed to facilitate printing. The vessel structures were positioned at the centre of the moulding area of the device and placed at only $100 \mu\text{m}$ distance from the mould's bottom plane to ensure that after moulding, the resulting chips would be compatible with high magnification microscopy. The arrangement of the vessel within the full 3D model was defined in such a way that the channel would be suspended and horizontally levelled to the Z-axis along the whole length of the device (Figure 1C). Any residual artefacts and mesh errors present in the 3D models were removed with Magics software (Materialise NV, Leuven, Belgium).

The full 3D models were then printed from PIC100 resin in a Perfactory 3 mini-multi lens SLA printer (EnvisionTEC GmbH, Gladbeck, Germany) using a resolution of $25 \mu\text{m}$ (Figure 1C). The resulting devices were flashed for 3500 cycles in an Otofash G171 apparatus (EnvisionTEC) and rinsed in 100% ethanol at 37°C for 7 hours, refreshing ethanol roughly every 2 hours. Using Fiji image analysis software [36], the resulting construct dimensions were determined. PDMS (Sylgard 184, Dow Corning, Midland MI, USA) pre-polymer was then mixed with the crosslinking agent in a 10:1 ratio, poured into the moulds and set to cure overnight at 60°C .

The chips were finished by first breaking open the moulds manually and then gently removing the vessel segments of the moulds by pulling with forceps and leaving an open channel inside the PDMS (Supplementary Figure A-1).

Finally, the chips were sterilized by means of plasma treatment (60 s at 100 W, 600 mTorr, Harrick Plasma) and bonded to a microscope cover glass.

3.2.3. Endothelial Cell Culture and Seeding

After the plasma treatment, the microfluidic channels in the chips were coated with a solution of 0.1 mg/ml rat tail collagen-I (Cultrex-Trevigen) in 1× Phosphate Buffered Saline (PBS; Sigma Aldrich). The collagen solution was introduced in the microfluidic device and incubated for 30 minutes followed by a PBS wash. The microfluidic device was endothelialised by introducing 40 µl of 4·10⁶ cell/ml HUVEC (Lonza) suspension in EGM-2 (Lonza) culture medium and incubating for 30 minutes on a platform rocker (Thermolyne Vari-mix) set to 6 rpm. The introduction of cells was repeated and the microfluidic device was incubated upside down for another 30 minutes. The endothelialised microfluidic device was cultured for a period of two days to ensure a confluent monolayer, during which the EGM-2 culture medium was refreshed twice per day.

3.2.4. Staining, Fluorescence Microscopy and Confocal Microscopy

A live/dead staining was performed using the Live/Dead Viability/Cytotoxicity Kit for mammalian cells according to the manufacturer's protocol (ThermoFisher Scientific – calcein AM, ethidium homodimer-1). Alternatively, to visualize cell morphology, the cells were fixated using 4% paraformaldehyde (Sigma Aldrich) solution and permeated using a 0.3% Triton X-100 (Sigma Aldrich) solution in PBS. The nuclei and F-actin filaments were stained using two molecular probes: NucBlue (ThermoFisher Scientific) and ActinGreen (ThermoFisher Scientific), respectively. The stained cells were imaged using an EVOS FL Cell Imaging System using DAPI, CY5 and GFP filter cubes. Confocal fluorescent microscopy was performed on a Nikon confocal A1 microscope with excitation lasers of wavelength 405 and 488 nm.

3.2.5. Blood Perfusion

Microfluidic devices were connected to a syringe pump (Harvard Apparatus PhD 2000) using custom connectors and Tygon tubing. The Tygon tubing was coated with a washing buffer of HEPES, 1% bovine serum albumin (Sigma

Aldrich), 1% glucose (Merck Millipore), 200 mM CaCl_2 (Sigma Aldrich) and 0.1 $\mu\text{L}/\text{mL}$ heparin (Sigma Aldrich) to avoid clotting. Human citrated whole blood was used in perfusion experiments. The blood was provided by the Experimental Centre for Technical Medicine (ECTM, MIRA Institute, University of Twente) and used within a maximum of 4 hours after donation. The citrated blood was re-calcified using a re-calcification buffer of 63.2 mM CaCl_2 and 31.6 mM MgCl_2 (ThermoFisher Scientific) and stained with 2 $\mu\text{g}/\text{mL}$ DiOC₆ (Life Technologies). Blood was pulled through the microfluidic device using the syringe pump at a flow rate of 0.29 mL/min yielding a shear rate of approximately 1000 s^{-1} . The chosen shear rate would recapitulate the shear rate experienced by endothelium in large arteries [37–39]. Platelet aggregation dynamics were quantified in Fiji. An image stack was generated with a resolution of 1 frame per second, capturing the full length of the perfusion. This stack was then converted to black/white images showing either the presence or absence of platelets, through specific thresholding. Finally, the platelets were quantified by measuring the white intensity (platelets) inside a region of interest (ROI) interpolated over the full image stack (Supplementary Figure A-8).

3.2.6. Computational Fluid Dynamics

COMSOL 5.1 Multiphysics CFD module was used to predict the fluid flow profile, streamlines and shear rate distribution inside the 3D vessel geometries. The laminar flow module, that assumes incompressible flow, no turbulence and does not neglect the inertial term in Navier-Stokes' law, was used. The CAD 3D vessel model that was incorporated in the chip design was also used in COMSOL using a straight section modelled with a vessel diameter of 400 μm , which is compared to the original CTA vessel geometry with a vessel diameter of 4 mm diameter. A flow rate of $4.83 \cdot 10^{-9} \text{ m}^3/\text{s}$ was set on the inlet for the miniaturized model, and a flow rate of $1.0 \cdot 10^{-6} \text{ m}^3/\text{s}$ was set for the original model, both resulting in a wall shear rate of 1000 s^{-1} . An entrance length of 1 m was chosen to provide a fully developed flow on entry of the geometry. The outlet pressure was set to atmospheric pressure with no back flow. On the outer boundary of the geometries, excluding the inlets and outlets, a no-slip boundary condition was imposed.

3.3. Results & Discussion

3.3.1. Fabrication of Microfluidic Chips with 3D Blood Vessel Geometries

The flow properties of vessels are greatly determined by their 3D shape. Therefore, to accurately mimic *in vivo* flow conditions in our model, it is crucial to closely replicate natural shapes. To attain exact *in vivo* shapes, human thoracic CTA scans were segmented and converted to 3D models, which were further isometrically reduced in size by a factor of 10 before being used to fabricate chips (Figure 1A-B).

It was found that only vessels with a diameter of approximately 100 μm would still 3D print successfully; by further reducing the diameter, the printed vessels would become fragile and would collapse during printing. Given this lower limit, we always printed the model vessels with a non-stenosis diameter of 400 μm , so that even a severe stenosis of 67% (stenotic diameter of approximately 130 μm) would still print successfully (Figure 1C-D). Even more severe occlusions could be printed by increasing the vessel diameter while keeping the stenotic diameter constant. Constructs with healthy vessel geometries showed a printing success rate of roughly 80%, while stenotic constructs showed a printing success of 50%. Printing defects consisted mainly of small offsets perpendicular to the vessel direction (Supplementary Figure A-2). In later experiments, such offsets would potentially alter flow patterns within the microfluidic chips and therefore the constructs possessing these defects were discarded. However, this productivity loss could be compensated by the fast production method and the number of chips that could be printed at once. All printed constructs also showed artefacts consisting of ring-like indentations (approximately 2 to 5 μm in size) that were the result of the partial overlap of consecutively printed layers. These artefacts are inherent to SLA at these resolutions and they were also found in the chips after moulding (Supplementary Figure A-3). Although these artefacts cannot be avoided, we did not see any indication of negative effects caused by these ridges (see sections below).

It was possible to observe that the successfully printed constructs closely matched the designed 3D model dimensions (Figure 1C). By using Fiji image analysis software, actual diameters of printed constructs were found to be 10 to

30 μm smaller than the design, with diameters of 370 μm in the straight channel segments, and 180 μm (55%) and 120 μm (67%) for the stenosis geometries.

When using the moulds in PDMS-based soft lithography, chips containing healthy and stenotic geometries could be successfully created. Both the stenotic and cylindrical geometries were easily removable from within the cured PDMS and did not damage the integrity of the chips whatsoever (Figure 1F). Although the vessel segments used in our studies were relatively straight, we also successfully used our method to produce chips with vessel segments containing bends with angles of up to 80° (Supplementary Figure A-4). The success rate of manufacturing chips with such dramatic curves is substantially lower due to entrapment of parts of the 3D-printed constructs inside the cross-linked PDMS. In order to produce chips with complex 3D curves, that more closely mimic *in vivo* shapes, success rates may be increased by using sacrificial resins [40] or modular chips [41].

The produced PDMS chips could be perfused with fluids, without any observation of leakage (Figure 1E). The created inlets and outlets allowed tight sealing of pipette tips and tube connectors and facilitated perfusion and hydrostatic pressure-driven flow between two pipette tips for cell culturing.

Unlike what is usually seen in conventional soft lithography techniques, within the proposed method, alignment of PDMS layers or bonding to glass is no longer needed to create channels [14,22,42]. Moreover, it was possible to create perfusable free-form 3D channels, rather than the typical square-section or stylized circular channels observed in standard PDMS microfluidic chips.

3.3.2. Endothelialisation of Microfluidic Chips with 3D Model Vessels

The engineered 3D microfluidic devices were endothelialised by culturing human umbilical vein endothelial cells (HUVECs) in the channels for two days. Since the PDMS could potentially still contain cytotoxic photo-crosslinker residues [43], which originated from the PIC100, a viability assay was performed on the microfluidic device. A live/dead staining of the endothelial monolayer showed that the number of dead cells was negligible (Supplementary Figure A-5). Confocal fluorescence microscopy and epifluorescence microscopy combined with nuclei and actin filament staining (Figure 2A-B) was used to analyse the morphology of the endothelial cells for a 1250 μm section of a microfluidic device.

The results (Figure 2C) confirmed that the endothelial cells had indeed formed a confluent monolayer and that the HUVECs possessed a typical cobblestone morphology. Furthermore, the inset (Figure 2C) demonstrated that the HUVECs were not affected by the microtopographical artefacts (Supplementary Figure A-3) that result from the SLA.

Figure 2. The HUVEC morphology in the 3D microfluidic chips. The nuclei and F-actin filaments of the HUVECs have been stained using NucBlue and ActinGreen respectively. (A,B) Confocal fluorescence microscopy images showing the top view and cross-section of the 3D vessel in a straight section of a channel of diameter 370 μm . Scale bar, 200 μm (C) Fluorescence microscopy image of the HUVECs near the outlet of the channel of 100 μm . Scale bar, 50 μm .

3.3.3. Computational Fluid Dynamics on the 3D Vessel Geometries

Shear rate distributions and fluid flow profiles in both the microfluidic 3D geometries and the original CTA scale 3D vessel geometries were analysed by

CFD. A comparison between healthy and stenotic geometries showed a difference in and near the stenotic area (Figure 3). Typical shear rates in the non-stenotic sections of the 3D vessel were in the range of 1000 s^{-1} . Because of the 67% occlusion, higher velocities (Figure 3B, D) and consequently shear rates (Figure 3A, C) were measured in stenotic sections with shear rates higher than 10000 s^{-1} . A comparison between the CTA scale geometry (Figure 3A-B) and the smaller microfluidic scale model (Figure 3C-D), showed that, with the chosen volumetric flow rate, the shear rate distribution in the stenosis is very similar, and this is also the case for higher shear rates. Shear rates higher than 5000 s^{-1} are considered as pathological and shear rates higher than $8000\text{--}10000\text{ s}^{-1}$ activate von Willebrand Factor [14,21]. Therefore, all shear rates above 10000 s^{-1} are considered as relevant. The CTA scale computational model showed an elevated shear rate downstream of the stenosis, which was a result of inertial forces in the curved section after the stenosis. Recirculation might have contributed to the different shear rate distributions found in the two stenotic models (Supplementary Figure A-6). The stenotic CTA scale model showed severe recirculation with elevated backflow velocities. The stenotic microfluidic scale model also showed recirculation, but the affected area and backflow velocities are smaller. The two healthy models did not show any sign of recirculation. The microfluidic scale model showed a different shear rate pattern because the Reynolds number in the CTA scale model is significantly higher in comparison to the microfluidic scale model, resulting in an altered shear rate distribution and recirculation.

To further investigate the effects of the aforementioned ring-like indentations, a smooth channel and a channel with $4\text{ }\mu\text{m}$ ridges, both with a diameter of $400\text{ }\mu\text{m}$, were compared using a CFD model. No significant differences in flow velocity profiles were found and shear rate distributions did show minor differences (Supplementary Figure A-7).

Figure 3. Finite element analysis of rheological parameters in healthy and stenotic vessels of 67% occlusion, simulated for CTA (A, B) and microfluidic (C, D) sized models. An input flow of 1 ml/min and a vessel diameter of 4 mm were used for the CTA sized models and an input flow of 0.29 ml/min and a vessel diameter of 400 μm for the microfluidic models (A, C) Wall shear rate simulation showed no shear increase in healthy (top) channels but shear exceeding 10000 s^{-1} was demonstrated in the stenotic (bottom) channels. (B, D) Similar dynamics can be seen in the flow velocity, increasing from 1 to 2.5 m/s (CTA scale) and 0.1 to 0.5 m/s (microfluidic scale) in the stenotic region. Scale bars (A, B), 2 mm. Scale bars (C, D), 200 μm .

3.3.4. Thrombosis-on-a-Chip

A comparison between healthy and stenotic geometries was made by perfusion of re-calcified citrated human whole blood through a series of endothelialised microfluidic devices with varying degrees of occlusion: 0%, 55% and 67%, respectively. Healthy and stenotic microfluidic devices were perfused at the same flow rates. The healthy geometries did not show any sign of thrombosis along the entire length of the microfluidic device, after at least 15 minutes of blood perfusion (Figure 4A-B). Furthermore, this demonstrated that the ring-like indentations did not influence platelet aggregation. The stenotic geometries did induce thrombosis at and downstream of the stenotic apices as demonstrated by clear fluorescent platelet aggregation (Figure 4C-D). The first signs of platelet

aggregation in the stenosis region could be observed as soon as two minutes after the start of whole blood perfusion.

Figure 4. Re-calcified citrated whole blood perfused, from left to right, at 1000 s^{-1} in microfluidic chips with endothelialised healthy and stenotic 3D geometries. Platelets have been stained using DiOC₆. (A, B) Healthy geometries after approximately 15 minutes of perfusion. There are no signs of platelet aggregation. (C, D) Stenotic geometry after 1 minute and 2.5 minutes of blood perfusion, respectively. Thrombosis is clearly seen at the apex of the stenosis. Scale bar, 200 μm .

Minor recirculation was expected based on the CFDs in the supplementary figures (Supplementary Figure A-6). Indeed, platelet aggregation was also observed in this recirculation zone distal to the stenotic geometries. Recirculation plays an important role in thrombus formation, as shear rate and exposure time increases [44]. Recirculation also results in a longer exposure time to paracrine activation factors and allows for easier deposition of fibrin in low flow zones because of a longer retention time [45,46]. Interestingly, the formation of a thrombus was not limited merely to the recirculation area, as platelet aggregation also occurred near the onset of the stenosis and at the apex of the stenosis where no recirculation is to be expected. The local increase in shear rate in these areas is

likely to also contribute to the observed thrombosis, for example by the activation of von Willebrand Factor resulting in platelet adhesion [14,15,47]. Additionally, by using re-calcified whole-blood, the intrinsic coagulation pathway will become active over the course of the experiment. This contact activation of coagulation will likely also contribute to the observed platelet aggregation because of the known cross-talk between activated coagulation factors and platelets [48]. Whole blood perfusion through healthy and stenotic geometries showed that thrombosis only occurred in microfluidic devices with a stenotic geometry, which suggests that – in line with previous reports [14,15] – fluid flow velocity gradients, minor recirculation, intrinsic coagulation pathways and increased shear rates are likely to be the determining factors of thrombogenesis in our microfluidic chips.

Although the constant flow rate used in this experiment is not completely representative of physiological flow patterns, it can be suitable to investigate the onset of thrombosis in a well-controlled setting. In future studies, a pressure-driven flow could be used instead, as this better mimics the dynamic changes in blood flow during vessel occlusion.

The thrombus size over time was determined by a semi-quantitative analysis in several microfluidic devices (Supplementary Figure A-8). Thrombus formation in 3 stenotic geometries was compared with a healthy geometry. Different aggregation mechanics are observed in the stenotic geometries. First, continuous aggregate growth which is characterized by a steady increase in thrombus size and intensity. Second, stick and slip growth which involves small aggregates to grow and dislodge, leading to an increase in intensity followed by a sharp drop in intensity after thrombus dislodgement. The stenotic geometries showed a combination of the aggregation mechanics although each experiment showed a different predominant mechanism.

3.4. Conclusions

By combining CTA data, SLA 3D printing and microfluidics, we have developed a new approach for manufacturing microfluidic *in vitro* cell culture models which closely mimic the architectures found in both healthy and stenotic arteries with high precision. With this approach thrombosis can be recapitulated in 3D vessel geometries in a way that is not possible in microfluidic chips fabricated with

typical 2D wafer-based soft lithography, or even in microfluidic chips produced with acrylic fibres or by advanced biofabrication methods [14,15,49]. Furthermore, because it is based on 3D models, this methodology allows for easy correlation and comparison of *in vitro* studies with *in silico* fluid flow simulations. In the future, the devices and method described herein could be used to employ medical CTA imaging data to produce patient-specific microfluidic chips, and as such, the method and proof-of-principle can be considered to be an important stepping stone towards patient-specific arterial thrombosis studies. Overall, our method represents an important step towards making microfluidic organ-on-chip-based technology increasingly relevant in experimental studies of arterial thrombosis.

3.5. References

1. United Nations World Health Organization, Fact sheet N°310 <http://www.who.int/mediacentre/factsheets/fs310/en/>. 310.
2. Jirouskova, M.; Shet, A.S.; Johnson, G.J. A guide to murine platelet structure, function, assays, and genetic alterations. *J. Thromb. Haemost.* **2007**, *5*, 661–9.
3. Suo, J.; Ferrara, D.E.; Sorescu, D.; Guldberg, R.E.; Taylor, W.R.; Giddens, D.P. Hemodynamic shear stresses in mouse aortas: implications for atherogenesis. *Arterioscler. Thromb. Vasc. Biol.* **2007**, *27*, 346–51.
4. Lemini, C.; Jaimez, R.; Franco, Y. Gender and inter-species influence on coagulation tests of rats and mice. *Thromb. Res.* **2007**, *120*, 415–419.
5. Cullen, P.; Baetta, R.; Bellosta, S.; Bernini, F.; Chinetti, G.; Cignarella, A.; Von Eckardstein, A.; Exley, A.; Goddard, M.; Hofker, M.; et al. Rupture of the atherosclerotic plaque: Does a good animal model exist? *Arterioscler. Thromb. Vasc. Biol.* **2003**, *23*, 535–542.
6. Badimon, L.; Vilahur, G.; Padro, T. Atherosclerosis and thrombosis: Insights from large animal models. *J. Biomed. Biotechnol.* **2011**, *2011*.
7. Furie, B.B.C.; Furie, B.B.C. Review series Thrombus formation in vivo. *J. Clinial Investig.* **2005**, *115*, 3355–3362.
8. Pérez, P.; Alarcón, M.; Fuentes, E.; Palomo, I. Thrombus formation induced by laser in a mouse model. *Exp. Ther. Med.* **2014**, *8*, 64–68.
9. Van Kruchten, R.; Cosemans, J.M.E.M.; Heemskerk, J.W.M. Measurement of whole blood thrombus formation using parallel-plate flow chambers - a practical guide. *Platelets* **2012**, *23*, 229–42.
10. Benam, K.H.; Dauth, S.; Hassell, B.; Herland, A.; Jain, A.; Jang, K.-J.; Karalis, K.; Kim, H.J.; MacQueen, L.; Mahmoodian, R.; et al. Engineered In Vitro Disease Models. *Annu. Rev. Pathol. Mech. Dis.* **2015**, *10*, 195–262.
11. Bhatia, S.N.; Ingber, D.E. Microfluidic organs-on-chips. *Nat. Biotechnol.* **2014**,

- 32, 760–772.
12. Meer, A.D. van der; Berg, A. van den Organs-on-chips: breaking the in vitro impasse. *Integr. Biol.* **2012**, 4, 461.
13. Meer, A.D. van der; Orlova, V. V.; Dijke, P. ten; Berg, A. van den; Mummery, C.L. Three-dimensional co-cultures of human endothelial cells and embryonic stem cell-derived pericytes inside a microfluidic device. *Lab Chip* **2013**, 13, 3562–3568.
14. Westein, E.; van der Meer, A.D.; Kuijpers, M.J.E.; Frimat, J.-P.; van den Berg, A.; Heemskerk, J.W.M. Atherosclerotic geometries exacerbate pathological thrombus formation poststenosis in a von Willebrand factor-dependent manner. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, 110, 1357–62.
15. Nesbitt, W.S.; Westein, E.; Tovar-Lopez, F.J.; Tolouei, E.; Mitchell, A.; Fu, J.; Carberry, J.; Fouras, A.; Jackson, S.P. A shear gradient-dependent platelet aggregation mechanism drives thrombus formation. *Nat Med* **2009**, 15, 665–673.
16. Westein, E.; De Witt, S.; Lamers, M.; Cosemans, J.M.E.M.; Heemskerk, J.W.M. Monitoring in vitro thrombus formation with novel microfluidic devices. *Platelets* **2012**, 23, 501–509.
17. Zhu, S.; Herbig, B.A.; Li, R.; Colace, T. V.; Muthard, R.W.; Neeves, K.B.; Diamond, S.L. In microfluidico: Recreating in vivo hemodynamics using miniaturized devices. *Biorheology* **2015**, 52, 303–318.
18. Tsai, M.; Kita, A.; Leach, J.; Rounsevell, R.; Huang, J.N.; Moake, J.; Ware, R.E.; Fletcher, D.A.; Lam, W.A. In vitro modeling of the microvascular occlusion and thrombosis that occur in hematologic diseases using microfluidic technology. *J. Clin. Invest.* **2012**, 122, 408–418.
19. Mannino, R.G.; Myers, D.R.; Ahn, B.; Wang, Y.; Margo Rollins; Gole, H.; Lin, A.S.; Guldborg, R.E.; Giddens, D.P.; Timmins, L.H.; et al. “Do-it-yourself in vitro vasculature that recapitulates in vivo geometries for investigating endothelial-blood cell interactions”. *Sci. Rep.* **2015**, 5, 12401.
20. Bark, D.L.; Ku, D.N. Platelet transport rates and binding kinetics at high shear over a thrombus. *Biophys. J.* **2013**, 105, 502–511.
21. Casa, L.D.C.; Deaton, D.H.; Ku, D.N. Role of high shear rate in thrombosis. *J. Vasc. Surg.* **2015**, 61, 1068–1080.
22. Xia, Y.; Whitesides, G.M. Soft Lithography. *Annu. Rev. Mater. Sci.* **1998**, 28, 153–184.
23. Zheng, Y.; Chen, J.; Craven, M.; Choi, N.W.; Totorica, S.; Diaz-Santana, A.; Kermani, P.; Hempstead, B.; Fischbach-Teschl, C.; López, J.A.; et al. In vitro microvessels for the study of angiogenesis and thrombosis. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, 109, 9342–7.
24. Zhang, Y.S.; Davoudi, F.; Walch, P.; Manbachi, A.; Luo, X.; Dell’Erba, V.; Miri, A.K.; Albadawi, H.; Arneri, A.; Li, X.; et al. Bioprinted thrombosis-on-a-chip. *Lab Chip* **2016**, 16, 4097–4105.

25. Han, S.; Yan, J.-J.; Shin, Y.; Jeon, J.J.; Won, J.; Eun Jeong, H.; Kamm, R.D.; Kim, Y.-J.; Chung, S. A versatile assay for monitoring in vivo-like transendothelial migration of neutrophils. *Lab Chip* **2012**, *12*, 3861.
26. Estrada, R.; Giridharan, G.A.; Nguyen, M.D.; Prabhu, S.D.; Sethu, P. Microfluidic endothelial cell culture model to replicate disturbed flow conditions seen in atherosclerosis susceptible regions. *Biomicrofluidics* **2011**, *5*.
27. Visser, J.; Peters, B.; Burger, T.J.; Boomstra, J.; Dhert, W.J. a; Melchels, F.P.W.; Malda, J. Biofabrication of multi-material anatomically shaped tissue constructs. *Biofabrication* **2013**, *5*, 035007.
28. Malda, J.; Visser, J.; Melchels, F.P.; Jüngst, T.; Hennink, W.E.; Dhert, W.J. a; Groll, J.; Hutmacher, D.W. 25th anniversary article: Engineering hydrogels for biofabrication. *Adv. Mater.* **2013**, *25*, 5011–28.
29. D 'urso, P.S.; Barker, T.M.; Earwaker, W.J.; Bruce, L.J.; Atkinson, R.L.; Lanigan, M.W.; Arvier, J.E.; Effeney, D.J. Stereolithographic biomodelling in cranio-maxillofacial surgery: a prospective trial. *J. Cranio-Maxillofacial Surg.* **1999**, *27*, 30–37.
30. Au, A.K.; Lee, W.; Folch, A. Mail-order microfluidics: evaluation of stereolithography for the production of microfluidic devices. *Lab Chip* **2014**, *14*, 1294–301.
31. Knowlton, S.; Yu, C.H.; Ersoy, F.; Emadi, S.; Khademhosseini, A.; Tasoglu, S. 3D-printed microfluidic chips with patterned, cell-laden hydrogel constructs. *Biofabrication* **2016**, *8*, 025019.
32. Zorlutuna, P.; Annabi, N.; Camci-Unal, G.; Nikkhah, M.; Cha, J.M.; Nichol, J.W.; Manbachi, A.; Bae, H.; Chen, S.; Khademhosseini, A. Microfabricated biomaterials for engineering 3D tissues. *Adv. Mater.* **2012**, *24*, 1782–1804.
33. Hull, C.W. Apparatus for production of three-dimensional objects by stereolithography. *U.S. Pat. no. 4,575,330* **1984**.
34. AGECANONIX: Cardiac and coronary artery CT from <http://www.osirix-viewer.com/resources/dicom-image-library/> (last checked 23-05-17).
35. Fedorov, A.; Beichel, R.; Kalpathy-Cramer, J.; Finet, J.; Fillion-Robin, J.C.; Pujol, S.; Bauer, C.; Jennings, D.; Fennessy, F.; Sonka, M.; et al. 3D Slicer as an image computing platform for the Quantitative Imaging Network. *Magn. Reson. Imaging* **2012**, *30*, 1323–1341.
36. Schindelin, J.; Arganda-Carreras, I.; Frise, E.; Kaynig, V.; Longair, M.; Pietzsch, T.; Preibisch, S.; Rueden, C.; Saalfeld, S.; Schmid, B.; et al. Fiji: an open-source platform for biological-image analysis. *Nat. Methods* **2012**, *9*, 676–682.
37. Dammers, R.; Stifft, F.; Tordoir, J.H.M.; Hamelers, J.M.M.; Hoeks, A.P.G.; Kitslaar, P.J.E.H.M. Shear stress depends on vascular territory: comparison between common carotid and brachial artery. *J. Appl. Physiol.* **2003**, *94*, 485–489.
38. Wu, S.P.; Ringgaard, S.; Oyre, S.; Hansen, M.S.; Rasmus, S.; Pedersen, E.M.

- Wall Shear Rates Differ between the Normal Carotid, Femoral, and Brachial Arteries: An In Vivo MRI Study. *J. Magn. Reson. Imaging* **2004**, *19*, 188–193.
39. Schmidt-Trucksass, A.; Schmid, A.; Brunner, C.; Scherer, N.; Zäch, G.; Keul, J.; Huonker, M.; Schmidt-Trucksäss, A.; Zäch, G. Arterial properties of the carotid and femoral artery in endurance-trained and paraplegic subjects. *J. Appl. Physiol.* **2000**, *89*, 1956–1963.
40. Liska, R.; Schwager, F.; Maier, C.; Cano-Vives, R.; Stampfl, J. Water-soluble photopolymers for rapid prototyping of cellular materials. *J. Appl. Polym. Sci.* **2005**, *97*, 2286–2298.
41. Vittayarukskul, K.; Lee, A.P. A truly Lego[®]-like modular microfluidics platform. *J. Micromechanics Microengineering* **2017**, *27*, 035004.
42. Huh, D.; Matthews, B.D.; Mammoto, A.; Montoya-Zavala, M.; Hsin, H.Y.; Ingber, D.E. Reconstituting organ level lung functions on a chip. *Science* (80-.). **2010**, *328*, 1662–1668.
43. Narayan, R.J. *Rapid Prototyping of Biomaterials: Principles and Applications*; 2014; ISBN 978-0857095992.
44. Poepping, T.L.; Rankin, R.N.; Holdsworth, D.W. Flow patterns in carotid bifurcation models using pulsed doppler ultrasound: Effect of concentric vs. eccentric stenosis on turbulence and recirculation. *Ultrasound Med. Biol.* **2010**, *36*, 1125–1134.
45. Rana, K.; Neeves, K.B. Blood flow and mass transfer regulation of coagulation. *Blood Rev.* **2016**, *30*, 357–368.
46. Jackson, S.P. Arterial thrombosis—insidious, unpredictable and deadly. *Nat. Med.* **2011**, *17*, 1423–1436.
47. Ruggeri, Z.M. The role of von Willebrand factor in thrombus formation. *Thromb. Res.* **2007**, *120*, 5–9.
48. Heemskerk, J.W.M.; Bevers, E.M.; Lindhout, T. Platelet activation and blood coagulation. *Thromb. Haemost.* **2002**, *88*, 186–193.
49. Jain, A.; van der Meer, A.D.; Papa, A.L.; Barrile, R.; Lai, A.; Schlechter, B.L.; Otieno, M.A.; Loudon, C.S.; Hamilton, G.A.; Michelson, A.D.; et al. Assessment of whole blood thrombosis in a microfluidic device lined by fixed human endothelium. *Biomed. Microdevices* **2016**, *18*, 1–7.

Chapter 4

Automated Analysis of Platelet Aggregation on Cultured Endothelium in a Microfluidic Chip Perfused with Human Whole Blood

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Abstract

Organ-on-a-chip models with incorporated vasculature are becoming more popular to study platelet biology. A large variety of image analysis techniques are currently used to determine platelet coverage, ranging from manually setting thresholds to scoring platelet aggregates. In this chapter, an automated methodology is introduced, which corrects misalignment of a microfluidic channel, automatically defines regions of interest and utilizes a triangle threshold to determine platelet coverages and platelet aggregate size distributions. A comparison between the automated methodology and manual identification of platelet aggregates shows a high accuracy of the triangle methodology. Furthermore, the image analysis methodology can determine platelet coverages and platelet size distributions in microfluidic channels lined with either untreated or activated endothelium used for whole blood perfusion, proving the robustness of the method.

4.1. Introduction

Cardiovascular diseases are the leading cause of death worldwide [1]. A lot of research has already been conducted to better understand, prevent and treat cardiovascular diseases. Animal models are frequently used as disease models [2], but often do not fully recapitulate human physiology [3,4]. Organ-on-a-chip models with integrated vascular compartments are promising *in vitro* alternatives that allow for control over used geometries and applied flow profiles. These models can be lined with human endothelial cells, only require small amounts of human whole blood for perfusion and, most importantly, allow for complex, dynamic aspects of cardiovascular diseases to be studied in an all-human setting [5–14].

Image analysis is an integral part of studying the behavior of cells in tissue culture *in vitro*, and multiple software packages exist to determine the confluency of a cell monolayer (with ImageJ) [15], track cell migration (with CellProfiler) [16], or monitor smooth muscle cells or cardiomyocyte contraction (with MUSCLEMOTION) [17]. Similarly, when incorporating blood-derived platelets and leukocytes into organs-on-chips, robust methods are needed for quantifying key descriptive indicators or characteristics, *e.g.*, rolling, adhesion, clotting time, platelet coverage and aggregate size distribution. Furthermore, a reliable image analysis technique suitable for organs-on-chips is required before these models can be used in personalized medicine applications or implemented in the drug development pipeline [18].

To quantify platelet adhesion in microfluidic channels or flow chambers, various strategies have been applied. When using fluorescently labeled platelets, the mean intensity of representative areas of a channel or chamber can be used as a measure of platelet coverage and aggregate growth [5,6,19]. However, this strategy only gives an averaged signal and does not allow for discrimination of single platelets or individual aggregates. In order to obtain data on individual platelets or aggregates, alternative strategies have been developed to manually select individual platelets in flow chambers [20] or to assign a morphological score to regions of interest (ROIs), differentiating between a few single adhered platelets to large platelet aggregates [21]. The downside of such methods is that they rely on manual selection and scoring, thereby limiting the throughput and leaving room for human error or bias. In order to automatically obtain

information on platelet coverage [8,11,21–24] and growth dynamics [22,23,25], a threshold will have to be applied to fluorescence microscopy data to differentiate platelet aggregates from background signals [8,11,21,24]. The level of the threshold can be set manually by a researcher to obtain the best signal-to-background ratio [21][25], but this again introduces issues related to the throughput and bias. The threshold can also be automatically determined using various automatic threshold techniques, *e.g.* mean, Otsu and triangle. These techniques determine a threshold by measuring the mean intensity (mean), minimizing intra-class variance in a bimodal histogram (with the Otsu method) [26] or by locating the base of a histogram peak (with the triangle method) [27]. Given the nature of the fluorescence microscopy data, with sparse areas of positive signal on a mostly negative background, resulting in a skewed unimodal histogram, the triangle method [27] stands out. Variations of this method have been applied successfully for platelet coverage analysis in collagen-coated flow chambers [22,23]. However, the method has not been applied to study platelet aggregation patterns in microfluidic channels lined by endothelial cells, which are an integral part of organs-on-chips.

When applying automated platelet analysis in microfluidic channels, edge effects have to be taken into account [28]. Low shear rates close to the channel edges might exacerbate fibrin deposition [8] and including these regions can therefore result in non-representative platelet coverage values. Moreover, endothelial cells in microfluidic channels can be either in a non-activated state which almost fully prevents platelet adhesion and aggregation, or in an activated state with dense platelet aggregates stabilized by fibrin [6,8,11]. It is unknown if the previously reported methods for automated analysis can be applied to reliably quantify platelet aggregation patterns in microfluidic channels lined by endothelial cells in activated and non-activated states.

In this chapter, we introduce an image analysis methodology to quantify platelet aggregate coverage and size distribution in microfluidic devices lined with activated and non-activated endothelial cells and perfused with human whole blood at arterial shear rates. We demonstrate that image analysis of unprocessed data can be automated by correcting channel misalignment, creating ROIs, extracting areas covered by platelet aggregates and determining their size distribution. Here, we show, for the first time, that this triangle methodology is suitable for the characterization of both non-activated and

activated endothelium cultured in microfluidic channels. Furthermore, the triangle methodology is compared to manual identification of single platelets and platelet aggregates and shows high degree of sensitivity, specificity and accuracy.

4.2. Materials and Methods

4.2.1. Microfluidic Device Fabrication

Polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning, Midland, MI, USA) channels were made using standard soft lithography techniques. First a mould was fabricated by patterning a negative photoresist (SU-8, Microchem, Round Rock, TX, USA) on a silicon wafer using photolithography. Then, a PDMS base and a curing agent were mixed at a 10:1 ratio (*w/w*) degassed, poured and baked at 60 °C for 4 h. The patterned PDMS slab was peeled off and trimmed, and its inlets and outlets, both 1 mm in diameter, were punched (Integra Miltex Biopsy Punch). The PDMS was bonded to a glass slide (Thermo Fisher Scientific, Waltham, MA, USA) using a plasma treatment (50 W, 50 kHz, 0.5 Torr for 40 s, CUTE MPR, Femto Science), which resulted in a sterile microfluidic channel of 51 $\mu\text{m} \times 300 \mu\text{m} \times 14 \text{ mm}$ (height $\times$ width $\times$ length, Figure 1B).

4.2.2. Endothelial Cell Seeding and Introduction of Inflammatory Cytokines

Directly after the plasma treatment, a 0.1 mg/ml collagen-I (Corning rat tail collagen-I, high concentration) solution was incubated at 37 °C for 30 min. Human umbilical vein endothelial cells (HUVECs, Lonza C2519A) were trypsinized (Gibco), centrifuged at 390 $\times g$ for 5 min and seeded twice at a seeding density of 15×10^6 cells/mL in endothelial cell growth medium (ECGM, Cell Applications, San Diego, CA, USA) to cover both the bottom and top of the microfluidic channel [8]. Cells were allowed to reach confluency before introducing the ECGM with (activated condition) or without (non-activated condition) 10–50 ng/ml tumor necrosis factor- α (TNF- α , Sigma Aldrich, St. Louis, MO, USA) and being incubated overnight. Images visualizing endothelial cell morphology were made by fixating the HUVEC monolayer with a 4% (*v/v*) formaldehyde (Thermo Fisher Scientific) solution followed by a permeabilization with 0.3% (*v/v*) Triton-X-100 (Sigma Aldrich, St. Louis, MO, USA). The nuclei and

actin filaments were stained by using NucBlue and ActinGreen (Thermo Fisher Scientific) and following the manufacturer's protocol, and imaged using DAPI and GFP filter cubes.

4.2.3. Whole Blood Perfusion

Using a syringe pump (Harvard PHD 2000 Syringe Pump), custom connectors and tubing (Tygon) ECGM perfusion was established at an arterial shear rate of 1000 s^{-1} . Human whole blood was provided by the Experimental Centre for Technical Medicine from the Technical Medical Centre, University of Twente, and used within 4 h after being drawn (3.2% sodium citrate, Vacuette, Greiner Bio-One). This research did not fall in the scope of the Dutch Medical Research Involving Human Subjects Act. In agreement with the Declaration of Helsinki, informed consent was obtained from all volunteers. Furthermore, the blood collection procedure was approved by the local medical research ethics committee (METC Twente). The platelets in the whole blood were stained using CD41-PE (1% (v/v), Thermo Fisher Scientific) or DiOC₆ (1 $\mu\text{g/mL}$, Thermo Fisher Scientific). After incubating the label, the citrated human whole blood was reconstituted by adding a re-calcification buffer (Gibco HEPES, 63.2 mM CaCl₂ from Sigma Aldrich, St. Louis, MO, USA and 31.6 mM MgCl₂ from Ambion). The re-calcified citrated whole blood with stained platelets was perfused at 1000 s^{-1} for 25 min. The volumetric flow rate was determined using the following equation:

$$\gamma = 6Q/wh^2$$

where γ is the shear rate (s^{-1}), Q is the volumetric flow (m^3/s), w is the channel width (m), and h is the channel height (m).

After the whole blood perfusion, the channel was washed with ECGM followed by a 4% (v/v) formaldehyde (Thermo Fisher Scientific, Waltham, MA, USA) wash to fixate the cells. Fluorescence microscopy images and phase contrast images were acquired using the EVOS FL imaging system (Thermo Fisher Scientific, Waltham, MA, USA). A $10\times$ magnification was used for all images. To measure platelet coverage in the microfluidic devices, an RFP filter cube was used when platelets were stained using CD41-PE and a GFP filter cube was used for samples stained with DiOC₆.

4.2.4. Determination of an ROI and Triangle Thresholding

Unprocessed sets of fluorescence microscopy images were imported in MATLAB (MathWorks), corrected for channel misalignment and two ROIs were created per figure. ROI #1 is the area spanned by the microfluidic channel and ROI #2 is the area that is located at least 50 μm from channel walls and figure borders. Using the triangle method, a threshold value was found in ROI #1 and applied in ROI #2, resulting in a black and white image where the white areas represent platelets or platelet aggregates. Platelet coverage was measured by calculating the ratio of white pixels versus black pixels. Platelet aggregate size distributions were determined by characterizing the white areas in the binary figures. Two scripts for determining platelet coverages and platelet aggregate size distributions have been released on the MATLAB central file exchange and can be found in the Supplementary Materials.

4.2.5. Manual Identification of Platelet Aggregates and Statistics

ROI #2 was manually processed by identifying individual platelets and platelet aggregates in MATLAB. The black and white images resulting from the manual identification of platelets and platelet aggregates were compared to the black and white images from the triangle methodology. A direct comparison resulted in true positives, false positives, true negatives and false negatives used to calculate sensitivity, specificity and accuracy. After calculating platelet coverages, using the same method as mentioned in Section 2.4, the platelet coverages found with the triangle methodology were plotted and compared to the platelet coverages found with the manual identification method. Furthermore, the R-squared value was determined by calculating squared residuals compared to $y = x$ using MATLAB.

4.2.6. Computational Fluid Dynamics

COMSOL Multiphysics 5.4 was used to conduct computational fluid dynamics modeling of the wall shear rate in the microfluidic device. A 2 mm long section of the microfluidic channel was modeled using the laminar flow module for incompressible flow. A no-slip boundary condition was imposed on all walls and a volumetric flow rate of 7.8 $\mu\text{L}/\text{min}$ was applied on the inlet, while the

atmospheric pressure was maintained on the outlet. Shear rate profiles were mapped on the three-dimensional (3D) model and the cutline data were exported to MATLAB for visualization.

4.3. Results

4.3.1. Microfluidic Device and Introduction of Data

Microfluidic chips were used for human whole blood perfusion experiments in endothelialized channels. A fluorescence microscopy image of a confluent monolayer of HUVECs is shown in Figure 1A (nuclei in blue and F-actin in green). The HUVEC monolayer was left untreated or exposed to TNF- α overnight to instigate inflammation. After 25 min of whole blood perfusion, the channels were rinsed and fixated. Figure 1 shows the used microfluidic chip and typical phase contrast and fluorescence microscopy images.

Both phase contrast data and fluorescence data can be used to measure platelet aggregation. For the phase contrast data, either the platelets or platelet aggregates have to be selected manually [20] or detected automatically using edge detection. Manually selecting adhered platelets (Figure 1C,E) and platelet aggregates is slow and prone to human error. The edge detection method should pick up not only single platelets but also platelet aggregates, which might have similar sizes and shapes compared to other particles like red blood cells, white blood cells, apoptotic cells and apoptotic bodies [29]. The use of fluorescence data (Figure 1D,F) is more robust and circumvents any accidental non-specific detection. A threshold can also be automatically determined by various automatic threshold techniques. In ImageJ (NIH Image) [15], 16 automated thresholds were qualitatively compared using a set of representative fluorescence microscopy images. The Otsu and triangle methods were the best at distinguishing platelets from the background. The Otsu method finds the threshold that minimizes the intra-class variance and works best with bimodal histograms [26]. However, minimal platelet adhesion is expected on non-activated endothelium, resulting in a unimodal histogram which makes the Otsu technique less suitable. The triangle method is a geometrical threshold method aimed at setting a threshold at the base of a histogram peak and works best for a skewed unimodal histogram [27]. The fluorescence microscopy images of

adhered platelets have a unimodal histogram, and therefore, the triangle method is suitable for determining the threshold.

Figure 1. Microfluidic chip and microscopy images. (A) Fluorescence microscopy image of a confluent monolayer of human umbilical vein endothelial cells (HUVECs) in a microfluidic chip (nuclei are in blue; F-actin is in green). (B) Render of the microfluidic chip used. Channels have dimensions of $51\ \mu\text{m} \times 300\ \mu\text{m} \times 14\ \text{mm}$ (height $\times$ width $\times$ length). (C,E) Phase contrast images of the microfluidic channel lined with endothelial cells after fixation and 25 min of whole blood perfusion. (D,F) Fluorescence microscopy image of platelets stained with CD41-PE and fixated after 25 min of whole blood perfusion. Insets in (E,F) show small platelet aggregates (large arrows) and single adhered platelets (small arrows). Scale bars, $100\ \mu\text{m}$.

4.3.2. Automated Threshold Using the Triangle Method

Fluorescence microscopy images were imported into MATLAB (version R2016b). To correct for misalignment of the microfluidic channel on the microscopy stage, the top edge of the channel was found by manually indicating the intersection of the top wall of the channel and the left and right borders of the figure (the top dashed line in Figure 2A). Using the coordinates of these intersections and the arctangent, the angle between the channel walls and the true horizontal line was

calculated and corrected using the “imrotate” function in MATLAB. Alternatively, the channel edge was found automatically by vertically scanning the image to find the first local maximum intensity followed by an angle sweep to determine the misalignment angle. Using the angle-corrected figure and the coordinates of the top channel wall extremities, two ROIs were cropped: ROI #1 spans the entire area inside the channel walls, and ROI #2 spans the area inside the channel at least 50 μm from the channel walls and image borders, as indicated in Figure 2B. Because rectangular channels were used, the flow rate close to the channel was affected by edge effects, resulting in lower wall shear rates. The area spanned by ROI#2 shows a flat wall shear rate profile and displays shear rates of $\geq 95\%$ of the maximum value (Supplementary Figure B-1). Therefore, the area located up to 50 μm from the channel wall was omitted in platelet coverage calculations.

Figure 2. Conversion of a grey-scale fluorescence microscopy image to a black and white image. (A) Raw data showing platelet aggregates in greyscale. The angle α indicates possible channel misalignment. (B) Angle-corrected and -cropped image. Dashed lines indicate the regions of interest. ROI #1 spans the entire microfluidic channel, and ROI #2 is the area of the microfluidic channel minus an outer rim of 50 μm . (C) Resulting black and white image after the triangle threshold was applied. (D) Schematic

representation of script operations and the resulting outputs. (E) Platelet aggregate size distribution. Scale bar, 100 μm .

The two ROIs were used to determine the platelet coverage and aggregate size distribution using the triangle threshold method. For the triangle threshold method to find a threshold that distinguishes adhered platelets from background, there need to be platelet aggregates. In the absence of foreground signals, the calculated threshold will shift and include a large proportion of the background signal, resulting in an exaggerated platelet coverage value. When analyzing microfluidic channels with healthy endothelium, little to no platelet adhesion was expected in ROI #2, while platelet aggregation can be expected in a 50 μm outer rim of ROI #1, due to edge effects. By using ROI #1 to determine the threshold and thus including aggregates formed in the proximity of the channel walls, as well as applying this threshold in ROI #2, this problem was circumvented.

The histogram of ROI #1 was used to determine the threshold by finding the maximum count and the artificial zero count (Figure 3A). This artificial zero was set by finding the first bin, in which the count was lower than 0.01% of the maximum count of the histogram. A linear line, the hypotenuse, was drawn from the artificial zero to the maximum count (Figure 3B). For each bin, the shortest distance between the hypotenuse and the histogram was calculated, which by definition was the length of a line orthogonal to the hypotenuse. The bin with the maximum distance between the histogram and the hypotenuse was set as the threshold (Figure 3C). Finally, the found threshold was imposed on ROI #2 (Figure 3D), resulting in a binary image where platelets and platelet aggregates are shown in white (Figure 2C).

Platelet coverage was determined by dividing the number of non-zero (white areas) values by the number of zero values $\times 100$. The binary images were used to investigate the aggregate size distribution by using the “regionprops” function in MATLAB.

Figure 3. Determination of the threshold in the fluorescence intensity histograms of platelet aggregation data with the triangle method. (A) Histogram of raw data with “bins” of fluorescence intensity on the x -axis and frequency on the y -axis. The maximum and the artificially set zero points are indicated. (B) The processed histogram in which the first bin coincides with the maximum count bin and the last bin coincides with the zero histogram bin. The orange line is the hypotenuse, connecting the maximum count bin in the histogram (“Max(h)”) and the zero histogram bin. The shortest distance between the histogram and the hypotenuse was measured for each bin, which by definition was the length of the line orthogonal to the hypotenuse. (C)

The resulting distances between the hypotenuse and histogram for each bin of the histogram. The bin with the maximum distance was chosen as the threshold. (D) Histogram with the triangle threshold applied.

4.3.3. Comparison of Binary Output Using the Triangle Method with the Manual Method

The proof of concept in Figure 2 shows that the triangle threshold is suitable for distinguishing platelets from the background. To determine the performance of this method in finding platelet aggregates, 10 fluorescence microscopy images were processed with the triangle threshold script and were compared to manually processed images. Higher-intensity versions of the fluorescence microscopy images were processed by manually drawing freeform lines around platelet aggregates, creating a mask representing the location and size of platelet aggregates.

The two black and white images (Figure 4B,C), produced with the triangle threshold and the manually drawn mask, show similarly shaped aggregates and also the measured platelet aggregate coverage is quite similar: 3.36% for the triangle threshold and 2.96% for the manual method. Both platelet aggregate size distributions were compared and displayed similar distributions (Supplementary Figure B-2). To determine how the triangle methodology compared to the manual identification, masks from both techniques were superimposed and a sensitivity of 84.84%, a specificity of 98.69% and an accuracy of 91.27% were determined. The triangle methodology recognized true positive values (sensitivity) and was even better at identifying true negatives (specificity). Furthermore, the triangle methodology was capable of detecting both true positives and true negatives, resulting in a high accuracy. The accuracy of each individual image is given in Figure 4D, showing high accuracies for untreated and activated endothelium. A comparison of the platelet coverages determined using the triangle methodology and manual identification is shown in Figure 4E, where the coordinates of manually determined coverages are plotted versus triangle-determined coverages ($R^2 = 0.9915$). Figures 4D-E show data generated with microfluidic channels lined with untreated and activated endothelium. The triangle method is capable of handling widely varying data ranging from almost no platelet coverage for untreated channels to occluded channels lined with

activated endothelium. This versatility shows the potential of using a triangle threshold methodology for the analysis of platelet aggregates.

To further illustrate the robustness of our method, we also analyzed fluorescence data that were obtained from experiments, in which the platelets were labeled with DiOC₆ instead of CD41-PE. DiOC₆ targeted mitochondria and, when added to a blood sample, stained endothelial cells over the course of the blood perfusion experiment. This resulted in an increase in background signal over time, but we demonstrated that patterns of DiOC₆ labeled platelets can also be successfully analyzed using the triangle method (Supplementary Figure B-3).

Figure 4. Comparison of fluorescence data of platelet aggregates processed using the automated method and manual identification. (A) Raw data showing platelet aggregates in greyscale. Dashed lines show the channel walls and ROI #2. ROI #1 spans the entire microfluidic channel, and ROI #2 is the area of the microfluidic channel minus the area with a 50 μm outer rim. (B) Binary image produced using the triangle method. (C) Binary image produced using the manual method. (D) Accuracy versus platelet coverage determined using the triangle methodology. Black dashed line represents mean accuracy. Both the activated (orange squares) and non-activated (yellow circles) endothelium are displayed. (E) Platelet coverage using the triangle method versus using the manual method. Black dashed function represents the line where the platelet coverage determined using the manual method correlates perfectly with the triangle method ($y = x$). Both the activated (orange squares) and non-activated (yellow circles) endothelium are displayed in the graph, showing a lower platelet coverage for the untreated endothelium in comparison to that for the inflamed endothelium. Scale bar, 100 μm .

4.4. Discussion

We have demonstrated that the triangle threshold method is a suitable way to analyze patterns of platelets in microfluidic channels lined with endothelial cells. The method correlates well with manual processing, detects platelet aggregates in a wide size range and is robust enough to deal with data with extremely low platelet coverage and high platelet coverage on healthy and activated endothelium, respectively.

The expected platelet adhesion patterns on endothelial cells range from single adhered platelets to 3D blood clots. The script only measures areas covered by platelets and thus the volumetric 3D platelet aggregates are collapsed onto an area. Using confocal microscopy might be of interest to determine whether this simplification is valid and might elucidate a link between platelet aggregate size and volume, adding to the descriptive power of the current methodology.

Others have calculated the platelet coverage only including thresholded areas larger than a single platelet [23]. Only microscopy images that are in the focus of the endothelial monolayer should be used, but because of the heterogeneous nature of the aggregates (small aggregates and bigger 3D aggregates), a large 3D aggregate might be partially out of focus, resulting in a slightly granular thresholded area. The out of focus area also represents an area covered by platelets and should be used in coverage measurements.

Studying platelet aggregation in flow chambers has been performed for decades and has led to important insights into platelet biology. Microfluidic channels coated with extracellular matrix proteins have helped understand bleeding and vascular injury [22,23]. Recently, organs-on-chips and their integrated blood vessels-on-chips are becoming increasingly popular to study platelet biology. These vessel-on-chip devices incorporate endothelial cells which opens up opportunity to study the interaction between endothelium and whole blood [5–14]. However, increased complexity goes hand in hand with challenges in analyzing data. The introduced methodology could be used by others to automatically determine platelet coverage and platelet aggregate size distributions in their organ-on-a-chip devices, even for data analysis of experiments with widely varying platelet coverages.

4.5. Conclusions

In this chapter, an automated analysis of platelet coverage and platelet size distribution was introduced for applications in organs-on-a-chips and vasculature-on-a-chip devices. The methodology corrects for channel misalignment, automatically defines ROIs and sets a threshold using the triangle method. The described method was compared to a manual identification method, where a user manually indicates adhered platelets and aggregates. Furthermore, a high sensitivity, a high specificity and a high accuracy were measured. The image analysis method presented here is capable of determining platelet coverages and platelet size distributions in microfluidic devices perfused with human whole blood lined with either activated or untreated endothelial cells, proving the robustness of the methodology.

4.6. Supplementary Materials

The automatic triangle threshold script and platelet size distribution script have been shared on the MATLAB central file exchange: Hugo Albers (2019). Automated Analysis of Platelet Aggregation in Microfluidics (<https://www.mathworks.com/matlabcentral/fileexchange/73348-automated-analysis-of-platelet-aggregation-in-microfluidics>), MATLAB Central File Exchange. Retrieved November 13, 2019.

4.7. References

1. WHO Fact Sheet - Cardiovascular diseases Available online: <https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-cvds> (accessed on Jul 15, 2019).
2. Zaragoza, C.; Gomez-Guerrero, C.; Martin-Ventura, J.L.; Blanco-Colio, L.; Lavin, B.; Mallavia, B.; Tarin, C.; Mas, S.; Ortiz, A.; Egido, J. Animal models of cardiovascular diseases. *J. Biomed. Biotechnol.* **2011**, *2011*, 497841.
3. Jirouskova, M.; Shet, A.S.; Johnson, G.J. A guide to murine platelet structure, function, assays, and genetic alterations. *J. Thromb. Haemost.* **2007**, *5*, 661–9.
4. Suo, J.; Ferrara, D.E.; Sorescu, D.; Guldberg, R.E.; Taylor, W.R.; Giddens, D.P. Hemodynamic shear stresses in mouse aortas: implications for atherogenesis. *Arterioscler. Thromb. Vasc. Biol.* **2007**, *27*, 346–51.
5. Costa, P.F.; Albers, H.J.; Linssen, J.E.A.; Middelkamp, H.H.T.; van der Hout, L.; Passier, R.; van den Berg, A.; Malda, J.; van der Meer, A.D. Mimicking arterial thrombosis in a 3D-printed microfluidic in vitro vascular model based on computed tomography angiography data. *Lab Chip* **2017**, *17*.
6. Westein, E.; van der Meer, A.D.; Kuijpers, M.J.E.; Frimat, J.-P.; van den Berg, A.; Heemskerk, J.W.M. Atherosclerotic geometries exacerbate pathological thrombus formation poststenosis in a von Willebrand factor-dependent manner. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 1357–62.
7. Zhang, Y.S.; Davoudi, F.; Walch, P.; Manbachi, A.; Luo, X.; Dell’Erba, V.; Miri, A.K.; Albadawi, H.; Arneri, A.; Li, X.; et al. Bioprinted thrombosis-on-a-chip. *Lab Chip* **2016**, *16*, 4097–4105.
8. Jain, A.; van der Meer, A.D.; Papa, A.-L.; Barrile, R.; Lai, A.; Schlechter, B.L.; Otieno, M.A.; Loudon, C.S.; Hamilton, G.A.; Michelson, A.D.; et al. Assessment of whole blood thrombosis in a microfluidic device lined by fixed human endothelium. *Biomed. Microdevices* **2016**, *18*, 73.
9. Jain, A.; Barrile, R.; van der Meer, A.D.; Mammoto, A.; Mammoto, T.; De Ceunynck, K.; Aisiku, O.; Otieno, M.A.; Loudon, C.S.; Hamilton, G.A.; et al. Primary Human Lung Alveolus-on-a-chip Model of Intravascular Thrombosis for Assessment of Therapeutics. *Clin. Pharmacol. Ther.* **2018**, *103*, 332–340.
10. Zheng, Y.; Chen, J.; Craven, M.; Choi, N.W.; Totorica, S.; Diaz-Santana, A.; Kermani, P.; Hempstead, B.; Fischbach-Teschl, C.; López, J.A.; et al. In vitro microvessels for the study of angiogenesis and thrombosis. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 9342–7.
11. Barrile, R.; van der Meer, A.D.; Park, H.; Fraser, J.P.; Simic, D.; Teng, F.; Conegliano, D.; Nguyen, J.; Jain, A.; Zhou, M.; et al. Organ-on-Chip Recapitulates Thrombosis Induced by an anti-CD154 Monoclonal Antibody: Translational Potential of Advanced Microengineered Systems. *Clin. Pharmacol. Ther.* **2018**, *104*, 1240–1248.

12. Mathur, T.; Singh, K.A.; R. Pandian, N.K.; Tsai, S.-H.; Hein, T.W.; Gaharwar, A.K.; Flanagan, J.M.; Jain, A. Organ-on-chips made of blood: endothelial progenitor cells from blood reconstitute vascular thromboinflammation in vessel-chips. *Lab Chip* **2019**, *19*, 2500–2511.
13. Menon, N.V.; Tay, H.M.; Wee, S.N.; Li, K.H.H.; Hou, H.W. Micro-engineered perfusable 3D vasculatures for cardiovascular diseases. *Lab Chip* **2017**, *17*, 2960–2968.
14. Mannino, R.G.; Myers, D.R.; Ahn, B.; Wang, Y.; Margo Rollins; Gole, H.; Lin, A.S.; Guldberg, R.E.; Giddens, D.P.; Timmins, L.H.; et al. “Do-it-yourself in vitro vasculature that recapitulates in vivo geometries for investigating endothelial-blood cell interactions”. *Sci. Rep.* **2015**, *5*, 12401.
15. Schneider, C.A.; Rasband, W.S.; Eliceiri, K.W. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **2012**, *9*, 671–675.
16. Carpenter, A.E.; Jones, T.R.; Lamprecht, M.R.; Clarke, C.; Kang, I.; Friman, O.; Guertin, D.A.; Chang, J.; Lindquist, R.A.; Moffat, J.; et al. CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biol.* **2006**, *7*, R100.
17. Sala, L.; van Meer, B.J.; Tertoolen, L.G.J.; Bakkers, J.; Bellin, M.; Davis, R.P.; Denning, C.; Dieben, M.A.E.; Eschenhagen, T.; Giacomelli, E.; et al. MUSCLEMOTION. *Circ. Res.* **2018**, *122*, e5–e16.
18. Probst, C.; Schneider, S.; Loskill, P. High-throughput organ-on-a-chip systems: Current status and remaining challenges. *Curr. Opin. Biomed. Eng.* **2018**, *6*, 33–41.
19. Six, K.R.; Devloo, R.; Van Aelst, B.; Vandekerckhove, P.; Feys, H.B.; Compennolle, V. A Microfluidic Flow Chamber Model for Platelet Transfusion and Hemostasis Measures Platelet Deposition and Fibrin Formation in Real-time. *J. Vis. Exp.* **2017**, e55351.
20. Dupuy, A.; Ju, L.; Passam, F. Straight Channel Microfluidic Chips for the Study of Platelet Adhesion under Flow. *BIO-PROTOCOL* **2019**, *9*.
21. de Witt, S.M.; Swieringa, F.; Cavill, R.; Lamers, M.M.E.; van Kruchten, R.; Mastenbroek, T.; Baaten, C.; Coort, S.; Pugh, N.; Schulz, A.; et al. Identification of platelet function defects by multi-parameter assessment of thrombus formation. *Nat. Commun.* **2014**, *5*, 4257.
22. Hansen, R.R.; Tipnis, A.A.; White-Adams, T.C.; Di Paola, J.A.; Neeves, K.B. Characterization of collagen thin films for von Willebrand factor binding and platelet adhesion. *Langmuir* **2011**, *27*, 13648–13658.
23. Neeves, K.B.; Onasoga, A.A.; Hansen, R.R.; Lilly, J.J.; Venckunaite, D.; Sumner, M.B.; Irish, A.T.; Brodsky, G.; Manco-Johnson, M.J.; Di Paola, J.A. Sources of Variability in Platelet Accumulation on Type 1 Fibrillar Collagen in Microfluidic Flow Assays. *PLoS One* **2013**, *8*.
24. Matsui, H.; Sugimoto, M.; Mizuno, T.; Tsuji, S.; Miyata, S.; Matsuda, M.; Yoshioka, A. Distinct and concerted functions of von Willebrand factor and

- fibrinogen in mural thrombus growth under high shear flow. *Blood* **2002**, *100*, 3604–10.
25. Colace, T.; Falls, E.; Zheng, X.L.; Diamond, S.L. Analysis of morphology of platelet aggregates formed on collagen under laminar blood flow. *Ann. Biomed. Eng.* **2011**, *39*, 922–929.
 26. Otsu, N. A Threshold Selection Method from Gray-Level Histograms. *IEEE Trans. Syst. Man. Cybern.* **1979**, *C*, 62–66.
 27. Zack, G.W.; Rogers, E.; Latt, S.A. Automatic Measurement of Sister Chromatid Exchange Frequency. *J. Histochem. Cytochem.* **1977**, *25*, 741–753.
 28. van der Helm, M.W.; van der Meer, A.D.; Eijkel, J.C.T.; van den Berg, A.; Segerink, L.I. Microfluidic organ-on-chip technology for blood-brain barrier research. *Tissue Barriers* **2016**, *4*.
 29. Hromada, C.; Mühleder, S.; Grillari, J.; Redl, H.; Holnthoner, W. Endothelial Extracellular Vesicles—Promises and Challenges. *Front. Physiol.* **2017**, *8*, 275.

Chapter 5

Human Induced Pluripotent Stem Cell-Derived Endothelial Cells in Microfluidic Channels Perfused with Human Whole Blood Enable Systematic Studies of Platelet Aggregation

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Abstract

Human induced pluripotent stem cell-derived endothelial cells (hiPSC-ECs) are used increasingly in experimental *in vitro* models for biomedical and pharmaceutical research. hiPSC-ECs have been shown to mimic many functional aspects of primary endothelium, *e.g.* barrier function and immune cell recruitment. However, hiPSC-EC-induced platelet aggregation has not yet been characterized. Here we demonstrate that hiPSC-ECs can be incorporated in a microfluidic thrombosis-on-a-chip. Channels were lined with hiPSC-ECs, pre-treated with the inflammatory cytokine tumor necrosis factor- α (TNF- α) and perfused with human whole blood, to study platelet aggregation. The aggregation response was similar to that found on inflamed human umbilical vein endothelial cells (HUVECs). Furthermore, channels lined with inflamed hiPSC-ECs perfused with whole blood and anti-platelet drug Eptifibatide resulted in significant reduction in platelet coverages. We have demonstrated that hiPSC-ECs are capable of supporting platelet aggregation and can be used to study endothelium-induced platelet aggregation in human whole blood in a well-controlled and systematic microfluidic assay.

5.1. Introduction

Worldwide 31% of all deaths are related to cardiovascular diseases (CVDs), of which 85% are due to heart attacks and strokes [1]. These acute forms of CVD are caused by arterial thrombosis, and therefore it is essential to further understand, prevent and treat this key pathophysiological process. Currently, a lot of research on thrombosis is conducted using animal models, and while these models can mimic the complex nature of cardiovascular diseases [2], there are clear differences between animals and humans in terms of platelet biology, genetics and arterial fluid dynamics [3,4]. Furthermore, it has been reported that results from animal model studies for drug development do not always translate well to humans [5–7], and therefore the development of realistic human *in vitro* models for thrombosis is of clear interest.

Traditional two-dimensional *in vitro* models incorporating human cells can be used to study thrombosis, but they do not fully recapitulate the key factors of the *in vivo* pathophysiological process. For example, thrombosis studies often require whole blood perfusion, which is impossible to perform in conventional well plate cultures. In contrast, microfluidic devices have been reported to allow perfusion of miniaturized versions of vasculature [8–17]. Furthermore, microfluidic ‘thrombosis-on-a-chip’ devices are a versatile tool since they allow for systematic control over geometry, flow rates and cell source, and they require relatively low volumes of blood.

Current vessel-on-a-chip models incorporate primary cells or immortalized cells of single donors or mixed populations. This lack of a realistic, standardized cell source leads to batch-to-batch variability and it misses a personalized approach [18,19], which can be essential in biomedical and pharmaceutical research. On the other hand, using primary material from specific patients is practically unfeasible. Alternatively, human induced pluripotent stem cells (hiPSCs) can be used to generate patient-specific cells in a controlled and scalable manner. A few years after the discovery of hiPSCs [20], robust differentiation protocols for generating endothelial cells (ECs) were described [21–27]. It has been reported that hiPSC-derived cells can lack maturity in comparison to primary cells, thus characterizing the inflammatory and pro-thrombotic response of hiPSC-ECs is of interest [18]. In recent works, the inflammatory properties of hiPSC-ECs have been compared to primary ECs [28], where hiPSC-ECs showed

a slightly less severe response to various inflammatory cytokines in comparison to human umbilical vein endothelial cells (HUVECs). Furthermore, a low batch-to-batch variability was reported when generating hiPSC-ECs using isogenic and non-isogenic hiPSCs [28]. Thus hiPSC-ECs are considered to be a promising candidate for a personalized approach in thrombosis studies.

Others have reported on a personalized approach for thromboinflammation studies before, using blood outgrowth endothelial cells (BOECs) [12]. Isolation and expansion of BOECs from a blood sample is quite time-consuming and takes four to six weeks. Furthermore, there is a high donor-to-donor variation in the number of BOEC colonies that can be derived. BOECs have a cobblestone-like morphology and are quite similar to vascular endothelial cells [29]. However, hiPSCs, as a source of endothelial cells, offer additional advantages, such as an unlimited supply of the cells from the same donor without donor-to-donor variation, possibility to introduce genetic modifications and scalable and reproducible production [18]. In addition, the use of hiPSCs could in the future aid in developing more complex *in vitro* models from a single donor, *e.g.* by also generating vascular smooth muscle cells (vSMCs) or pericytes from the same donor and incorporating them in the same model [30].

Here, we demonstrate that hiPSC-ECs can induce platelet aggregation in flowing human whole blood when cultured in a microfluidic channel. In addition, we demonstrate that the aggregation response can be inhibited by clinically relevant concentrations of the anti-platelet drug Eptifibatide. We expect this thrombosis-on-a-chip assay based on hiPSC-ECs will enable more accurate and systematic biomedical and pharmaceutical studies in the field of thrombosis.

5.2. Materials & Methods

5.2.1. Chip Fabrication

A mold for soft lithography was fabricated using standard photolithography techniques. First a silicon wafer was patterned with a SU-8 (Microchem) layer, by spin-coating negative photo resist followed by local UV exposure using a mask, post-baked and developed, which resulted in a mould with 51 μm high features representing the microfluidic channels. Polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning) was mixed, at a 1:10 ratio (w/w) curing agent to pre-polymer, degassed, poured on the mould and baked for 4h at 60 °C. The microfluidic

devices were cut to size and 1 mm diameter inlets and outlets were punched using a biopsy punch (Integra Miltex). The open PDMS chips were bonded to glass microscopy slides (ThermoScientific) using a plasma treatment (CUTE, Femto Science, 50 W, 50 kHz, 40 seconds exposure). Immediately after the plasma treatment the microfluidic channels were coated with 0.1 % (w/v) collagen-I (Rat tail, Corning) and incubated for 30 minutes.

5.2.2. Cell Culture

Human induced pluripotent stem cell-derived endothelial cells (hiPSC-ECs) were provided by the Leiden University Medical Center (LUMC, FLB243#6, CD31+, DIF#184 P1) and were differentiated using previously reported protocols [21,22].

Human skin fibroblasts were reprogrammed to hiPSCs using a non-integrative Sendai Virus [31,32] and redirected to endothelial fate by exposing the hiPSCs to medium containing bone morphogenetic protein 4, activin A and vascular endothelial growth factor (VEGF) followed by culture in VEGF and transforming growth factor- β inhibitor (SB431542) supplemented medium. Using magnetic CD31-specific dynabeads (Invitrogen) only differentiated ECs were extracted from the heterogeneous culture. The expanded ECs were frozen in dimethyl sulfoxide and fetal calf serum supplemented medium to be thawed upon use.

HUVECs (Lonza, C2519A) were cultured in ECGM (Cell Applications) on collagen coated T75 (Cellcoats, Greiner Bio One) until passage 6. HiPSC-ECs were expanded in Serum-Free Medium (SFM, Gibco) supplemented with 1% platelet poor plasma (Bovine, Bio-Techne), 30 ng/ml VEGF (Recombinant Human VEGF 165, R&D Systems) and 20 ng/ml bFGF (Human FGF-2, Miltenyi Biotec) on T75 coated with 0.1% (w/v) gelatin (Porcine skin type A, Sigma Aldrich) solution until passage 3. When 80-90% confluency was reached HUVECs and hiPSC-ECs were disassociated using trypsin (Thermo Fisher Scientific) and tryple (Gibco) respectively. Both ECs were centrifuged for 5 minutes at 390g and the pellet was re-suspended in ECGM resulting in a final concentration of 15×10^6 cells/ml. The microfluidic channels were lined with ECs by introducing 5 μ l of cell suspension followed by an incubation step of 30 minutes. During incubation, the disassociating of a second batch of cells was performed, in order to line the top

of the channel, which was achieved by incubating the microfluidic chip upside-down.

After the HUVECs and hiPSC-ECs had reached confluency, a 10 ng/ml TNF- α (Sigma Aldrich) in ECGM solution was introduced in the microfluidic channels to induce inflammation while ECGM was introduced in the control conditions and also incubated for 16h.

5.2.3. Whole Blood Perfusion

Citrated human whole blood was provided by the Experimental Centre for Technical Medicine of the TechMed Centre at the University of Twente in 3.5 ml citrate vacuettes (Greiner Bio One) and used within 4h of being drawn. Prior to whole blood perfusion the platelets were stained using CD41PE (1% v/v, ThermoFisher) and fluorescently labeled fibrinogen (10 μ g/ml, Alexa Fluor 647, Invitrogen) was added. Drug studies required the addition of 2 μ g/ml Eptifibatide (Tocris) which was added simultaneously with the labels 10 minutes prior to whole blood perfusion. The citrated whole blood and labels were re-calcified using 10% (v/v) of re-calcification buffer (HEPES, Gibco, 63.2 mM CaCl₂, Sigma Aldrich, 31.6 mM MgCl₂, Ambion). Two minutes prior to the introduction of whole blood, a shear rate of 1000 s⁻¹ was applied using a syringe pump (Harvard PhD 2000) to ensure an established flow. A pipette tip with 200 μ l of re-calcified citrated whole blood was attached to the inlet starting the whole blood perfusion at a set shear rate of 1000 s⁻¹, a microfluidic chip perfused with whole blood is shown in Figure 1B. Whole blood was perfused for 25 minutes, followed by a 1 minute wash using ECGM and a fixation using 4% paraformaldehyde (v/v, Sigma Aldrich) for 30 minutes at room temperature.

5.2.4. Fluorescent Labeling and Immunostaining

Before staining cells, they were fixated using 4% paraformaldehyde (v/v, Sigma Aldrich) for 30 minutes followed by permeabilization using 0.3% Triton-X (v/v, Sigma Aldrich) for 30 minutes. Various combinations of the following stainings were used for morphology studies NucBlue, ActinGreen & ActinRed (3 drops/ml). For VE-cadherin staining, a rabbit primary anti-body (1:100, 4h at 37°C, anti-VE-cadherin, polyclonal, Abcam) and secondary goat anti-rabbit (1:100, 1h at 20 °C, polyclonal, Alexa Fluor 488, Abcam) were used. ICAM-1 was

stained using a primary mouse antibody (1:100, 4h at 37°C, anti-ICAM-1 G-5, Santa Cruz) in combination with a secondary goat anti-mouse antibody (1:50, 1h at 20 °C, Alexa Fluor 488, Invitrogen). Platelets were stained using CD41PE (1% v/v), and fibrin was visualized by adding 10 µg/ml labeled fibrinogen (Alexa Fluor 647, from human plasma, Invitrogen) to whole blood 10 minutes prior to whole blood perfusion.

5.2.5. Microscopy and Image Analysis

Phase contrast and fluorescence microscopy images were made using EVOS imaging systems using 10× objective and DAPI, GFP, RFP and CY5 filter cubes. Fluorescence microscopy images were automatically processed using a custom MATLAB script [33]. The script corrected any possible channel misalignment and automatically determined the platelet coverage. A second custom MATLAB script was used to characterize platelet aggregate size distributions (both scripts can be found on the MATLAB central file exchange <https://www.mathworks.com/matlabcentral/fileexchange/73348-automated-analysis-of-platelet-aggregation-in-microfluidics>).

First raw data was corrected for skewness and two regions of interest (ROIs) were determined. A threshold was found in the ROI spanning the entire channel using the Triangle methodology [33–37], and was then applied on a ROI that spans the microfluidic channel excluding a 50 µm wide zone next to channel walls and image borders. This zone was excluded to account for edge effects which resulted in non-uniform shear rates [33]. The first MATLAB script produces a black and white image of the inner ROI, where white areas represent platelets and aggregates. Platelet coverage percentage was determined by measuring the amount of non-zero pixels and comparing this to the total amount of pixels. The second MATLAB script used this black and white image to determine platelet aggregate size distributions.

Nuclear orientation was determined by applying a threshold to the channel representing the stained nuclei. The MATLAB function ‘regionprops’ can determine a nucleus’ angle compared to the horizontal by fitting an ellipsoid and determining the angle between the major axis and the horizontal.

5.2.6. Statistics

Normality of platelet coverages and platelet aggregate size populations was tested using Kolmogorov-Smirnov (Origins). Two-tailed student's t-test ($p < 0.05$, MATLAB) was used to determine whether platelet coverage and platelet aggregate size populations were significantly different.

Platelet aggregate size distributions for HUVECs and hiPSC-ECs have a different number of replicates and different number of fluorescence microscopy images. Distributions were normalized by dividing the count in each bin by the number of microscopy images for that specific microfluidic channel. To prevent counts lower than one, all bins were multiplied with the highest number of microscopy images taken for one microfluidic channel. Effectively creating a scaled platelet aggregate size distribution that shows platelet aggregate count per surface area.

The determined nuclear orientations were normalized to account for a different number of counted nuclei, in such a way that the cumulative length of all bins in each condition is equal to one (MATLAB probability normalization).

5.3. Results

5.3.1. Human Endothelial Cells Cultured in Microfluidic Channels Form Monolayers and can be Stimulated with Inflammatory Cytokines

We designed a microfluidic chip that contains multiple microfluidic channels with a cross-section of $300 \times 51 \mu\text{m}$ (width $\times$ height) and a length of 14 mm (Figure 1A-B). Chips were fabricated from polydimethylsiloxane (PDMS), a silicone rubber typically used for microfluidic cell culture assays. HUVECs and hiPSC-ECs were seeded in microfluidic channels at various seeding densities, ranging from $5 - 20 \times 10^6$ cells/ml. The optimal seeding density was 15×10^6 cells/ml, which resulted in a confluent monolayer after 5 - 6h of incubation and did not show any cell detachment due to over-seeding (Supplementary Figure C-1). To induce inflammation the ECs were exposed to 10 ng/ml TNF- α for 16h which is in line with concentrations reported in literature [17,28,38].

In this study the inflammatory and pro-thrombotic properties of hiPSC-ECs and HUVECs were compared. Besides causing inflammation, TNF- α can also

induce apoptosis, which exposes a collagen-I substrate and could instigate platelet aggregation [39]. Since the goal of this study is to investigate the interplay between activated ECs and whole blood and not the adhesion of platelets to collagen, we verified that the seeding protocol and the chosen concentration of TNF- α resulted in confluent monolayers in untreated and inflamed conditions (Figure 1C).

Figure 1. Microfluidic channels lined with confluent monolayers of endothelial cells for whole blood perfusion. (A) Render of a microfluidic chip for thrombosis studies including channel dimensions. (B) Photograph of chip with connected whole blood reservoir and tubing. (C) Confluent monolayers of HUVECs and hiPSC-ECs cultured in a microfluidic device in control and inflamed conditions. Nuclei in blue and VE-cad in green. Flow from left to right. Scale bar, 100 μm .

Figure 1C shows the morphology (nuclei in blue, VE-cadherin in green) of HUVECs and hiPSC-ECs in control and inflamed, TNF- α treated, conditions. Both cell types showed confluent monolayers in both conditions. However, HUVECs displayed a cobble stone-like morphology while hiPSC-ECs showed a more elongated morphology. VE-cadherin staining showed a tendency of the hiPSC-ECs to align parallel to the channel walls, while the HUVECs displayed a more random orientation. A measure for cellular polarity of ECs is nuclear

orientation [40–42]. HUVECs displayed a more chaotic nuclear orientation in comparison to hiPSC-EC (Supplementary Figure C-2) which is in line with the cytoskeletal directionality in Figure 1.

Exposure of ECs to TNF- α induces upregulation of pro-adhesive receptors on their surface, *e.g.* intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), etc. [43], allowing adhesion of white blood cells. Furthermore, ICAM-1 is an important mediator of platelet aggregation on the surface of the inflamed endothelium [44–47]. Platelet adhesion to stimulated endothelium occurs indirectly through the binding of platelet integrin $\alpha_{IIb}\beta_3$ to ‘bridging molecules’, *e.g.* von Willebrand Factor (vWF), fibrinogen or fibronectin, which are attached to endothelial $\alpha_v\beta_3$ -integrins, glycoprotein Ib α or ICAM-1 [45,47]. Supplementary Figure C-3 shows the HUVECs and hiPSC-ECs stained for ICAM-1 with a nucleus and F-actin counterstaining. The ECs in microfluidic channels exposed to TNF- α expressed ICAM-I while HUVECs and hiPSC-ECs in the control condition showed a lower ICAM-1 expression, in line with literature [17,28].

5.3.2. Platelet Aggregates Form on Inflamed Endothelium in the Microfluidic Channels upon Whole Blood Perfusion at Arterial Shear Rates

After characterizing the morphology of the ECs in microfluidic channels, the pro-thrombotic properties of both cell types were compared. Microfluidic channels lined with either HUVECs or hiPSC-ECs in activated or inactivated state were perfused with human re-calcified citrated whole blood at 1000 s⁻¹ for 25 minutes. Healthy untreated endothelium strives to prevent the triggering of haemostatic responses, to maintain a healthy blood flow, while inflamed endothelium expresses various surface markers and tissue factor [39] to recruit white blood cells and activate and bind platelets. Thus 25 minutes of whole blood perfusion in chips, lined with untreated HUVECs or hiPSC-ECs should show minimal platelet adhesion while the inflamed endothelium is expected to cause increased platelet adhesion and aggregation [48].

Figure 2. Cultured human endothelium induced platelet aggregation and fibrin formation in human whole blood. (A) End-point measurements of microfluidic channels lined with either HUVECs or hiPSC-ECs after 25 minutes of human recalcified citrated whole blood perfusion at 1000 s^{-1} . Microfluidic channels lined with untreated or inflamed endothelium were perfused with whole blood in the control and inflamed conditions while $2 \text{ }\mu\text{g/ml}$ Eptifibatide was added to whole blood for the inflamed + condition. Inset shows large fibrin-rich platelet aggregates on inflamed endothelium. Platelets in orange and fibrin in cyan. Flow from left to right. Scale bars, $100 \text{ }\mu\text{m}$.

Figure 2 shows endpoint measurements where platelet adhesion (orange) and fibrin formation (cyan) are visualized. The control conditions for both HUVECs and hiPSC-ECs showed almost no platelet coverage while the inflamed conditions showed fibrin-rich platelet aggregates. In the control conditions some platelet aggregation was observed close to the channel walls due to edge-effects creating lower shear regions, thus allowing platelet aggregation. In channels exposed to $\text{TNF-}\alpha$ more platelet aggregation was observed and no clear difference between HUVECs and hiPSC-ECs was seen. To quantify platelet coverage a region of interest was defined by excluding the outer $50 \text{ }\mu\text{m}$ of the microfluidic channel where wall shear rates $<95\%$ of the maximum shear rate. The platelet coverage was determined in this region of interest by application of a triangle threshold script [33–37].

Figure 3. Platelet coverage and distribution of aggregate sizes in microfluidic channels lined with hiPSC-ECs and HUVECs in untreated or inflamed conditions. Furthermore, the effect of a clinically relevant concentration of the anti-platelet therapeutic Eptifibatide was shown on inflamed hiPSC-ECs. (A) Platelet coverage and count / area of regimes III and IV, comparing the control conditions (n = 5 for HUVECs, n = 6 for hiPSC-ECs), inflamed conditions (n = 11 for HUVECs, n = 12 for hiPSC-ECs) and inflamed + Eptifibatide conditions (n = 5 for hiPSC-ECs). (B) Examples of regimes used to describe platelet coverage, ranging from single adhered platelets (I, 1 – 10 μm^2), small aggregate (II, 10 - 10² μm^2), intermediate platelet aggregate (III, 10² - 10³ μm^2) and large platelet aggregate (IV, >10³ μm^2). (C) Ratio of mean counts / area of platelets and platelet aggregates in each regime. Error bars represent standard error of the mean and differences in populations were determined using two-tailed student's t-test (p<0.05). Scale bar, 100 μm .

Figure 3A shows platelet coverages and aggregate counts of regime III and IV, where examples of the regimes are given in figure 3B. Each regime contains a biologically relevant range of platelet aggregate sizes defined as: single platelets (I, 1 – 10 μm^2), small aggregates (II, 10 - 10² μm^2), intermediate aggregates (III, 10² – 10³ μm^2) and large aggregates (IV, >10³ μm^2). The adhesion and subsequent release of single platelets and small aggregates (regimes I & II) is less likely to result in an embolism in comparison to the release of bigger and often fibrin-rich

blood clots (regimes III & IV). Therefore, only counts / area in regimes III and IV were displayed in figure 3A. A significant difference was found between platelet coverages of the healthy and inflamed conditions for both HUVECs and hiPSC-ECs (two-tailed student's t-test, $p < 0.05$). Control conditions of HUVECs ($n = 5$) and hiPSC-ECs ($n = 6$) resulted in platelet coverages of 1.39% and 1.63% respectively, inflamed conditions resulted in platelet coverages of 3.08% and 4.57% for HUVECs ($n = 11$) and hiPSC-ECs ($n = 12$) respectively. No significant differences between HUVECs and hiPSC-ECs were found in the respective conditions.

The aggregate size distributions of the four regimes (Figure 3C) show a clear difference between control and inflamed conditions for HUVECs and hiPSC-ECs, it can be observed that the majority of counts / area is measured in the lowest regime. Furthermore, the counts / area are higher for hiPSC-ECs in comparison to HUVECs regardless of the applied condition. The displayed counts in regime III and IV show similar trends compared to the platelet coverages found in figure 3A. For both inflamed conditions the amount of platelet aggregates in regime III was significantly higher (two-tailed student's t-test, $p < 0.05$) than in control conditions. In regime IV only hiPSC-ECs showed a significant difference (two-tailed student's t-test, $p < 0.05$) between untreated and inflamed conditions.

5.3.3. Platelet Aggregation on hiPSC-ECs in Microfluidic Channels can be Inhibited by Pre-Treatment of Whole Blood with a Clinical Anti-Platelet Compound

Due to their donor-specific nature, implementation of hiPSC-ECs in microfluidic disease models could greatly advance personalized medicine approaches or could be used as an early predictor of human response in drug discovery pipelines [18]. Consequently, as a proof-of-principle, microfluidic channels lined with hiPSC-ECs were used to study the effect of the antiplatelet therapeutic drug Eptifibatide (also marketed as Integrilin®) at a clinically relevant concentration of 2 $\mu\text{g}/\text{ml}$. Eptifibatide is an effective platelet inhibitor by blocking glycoprotein IIb/IIIa or integrin $\alpha_{\text{IIb}}\beta_3$ receptors inhibiting platelet adhesion to fibrinogen and von Willebrand factor (vWF) [49]. In a clinical setting a steady state concentration of 1.5-2.2 $\mu\text{g}/\text{ml}$ in whole blood is advised [50–52] which results in the inhibition of approximately 80% of glycoprotein IIb/IIIa sites [50]. In microfluidic channels

lined with endothelial cells and exposed to TNF- α , fibrin and vWF will still offer binding sites for platelets but the majority of glycoproteins on the platelet surface are blocked resulting in reduced platelet aggregation [13].

Platelet coverages and counts / area of regime III and IV of hiPSC-ECs in untreated control conditions were compared to inflamed hiPSC-ECs perfused with either normal whole blood or whole blood with Eptifibatide. End-point measurements of microfluidic chips lined with inflamed hiPSC-ECs perfused with human whole blood showed more platelet aggregation in comparison to inflamed hiPSC-ECs perfused with whole blood and Eptifibatide (Figure 2). This is confirmed by the platelet coverages in Figure 3A, where the Eptifibatide condition (1.38%, $n = 5$) showed similar platelet coverages compared to the control condition (1.63%, $n = 6$). Both of these conditions showed significantly lower platelet coverages compared to the inflamed condition (4.57%, $n = 12$) without Eptifibatide (two-tailed student's t -test, $p < 0.05$).

Platelet aggregate size distributions show a difference between microfluidic channels lined with inflamed hiPSC-ECs perfused with whole blood with and without Eptifibatide (Figure 3C). Across the board, higher counts were measured for the inflamed condition without Eptifibatide. A comparison between the control and Eptifibatide conditions showed higher counts for the control condition, especially in the smaller aggregate size range, resulting in a slightly higher platelet coverage in the control condition. A comparison between inflamed and Eptifibatide conditions showed significantly lower counts / area (two-tailed student's t -test, $p < 0.05$) for whole blood perfusion with Eptifibatide in regime I and IV.

The proof-of-principle presented in Figures 2 and 3 showed that on inflamed hiPSC-ECs, a significant reduction in platelet aggregation could be achieved by the introduction of a clinically relevant concentration of Eptifibatide. This demonstrates that a thrombosis-on-a-chip model that incorporates hiPSC-ECs can in principle be used for the testing of therapeutics.

5.4. Discussion

Currently six channels lined with hiPSC-ECs can be perfused per experimental session. The amount of chips used for an experiment can quite easily be increased, but setting up whole blood perfusion in multiple devices is

challenging. Therefore, designing a chip with multiplexed channels or using a flow divider suitable for whole blood perfusion can increase the data output and thus significance of these measurements.

The hiPSC-ECs that were used in this study were generated using one control donor. Ideally, multiple donors would be used to investigate the robustness of the differentiation protocol. We have previously shown that hiPSC-ECs derived from independent control or healthy hiPSC lines are comparable [28]. In addition, barrier function, inflammatory responses and leukocyte adhesion were compared and little batch-to-batch variability was found. Thus, similar results can be expected when repeating thromboinflammation experiments using hiPSC-ECs derived from healthy controls. At the same time our assay likely will also allow to capture individual differences due to different genetic backgrounds or presence of various genetic conditions, and it would be interesting to test this in the future.

One of the advantages of utilizing hiPSC-ECs is the possibility to generate large number of donor-specific cells with minimally invasive procedures. It would be of interest to reprogram skin fibroblasts of both healthy and diseased individuals (*e.g.* von Willebrand disease) to be used in microfluidic models. Furthermore, not only generating ECs but also smooth muscle cells would increase the complexity of these thrombosis models. Additionally, it would be more realistic to utilize the generated hiPSC-ECs in combination with the donor's whole blood.

The research presented in this chapter is focused on platelet aggregation. Inflamed ECs upregulate ICAM-1 expression, which plays a role in the adhesion of activated platelets but also the adhesion of white blood cells. Others have investigated and quantified leukocyte adhesion to inflamed hiPSC-ECs [28]. The introduced devices could also be used to study leukocyte adhesion on inflamed hiPSC-ECs during whole blood perfusion.

In this study an arterial shear rate of 1000 s^{-1} was applied to mimic arterial thrombosis. Alternatively, different flow could be applied resulting in either a venous or pathological flow shear rate. The condition with a low venous-like shear rate could be used in conjunction with hiPSC differentiated to a venous EC phenotype. The flexibility of choosing an appropriate shear rate makes this system suitable for simulating either venous or arterial thrombosis.

Although others have reported that PDMS is not ideal for drug testing [53], since small molecules can adsorb onto the PDMS surface the required concentrations might differ from actual drug concentrations. The PDMS devices used were lined with ECs, creating a tight confluent monolayer. So initially TNF- α concentrations should not change due to adsorption to PDMS. Whole blood with Eptifibatide is perfused at arterial shear rates through the microfluidic channel, where the average residence time of whole blood being perfused through the chip is less than 2 seconds. While the refresh rate of Eptifibatide in the microfluidic channel is high, the therapeutic agent is exposed to whole blood for 10 minutes prior to perfusion. In clinical studies up to 25% of Eptifibatide can bind to plasma *in vivo*. This means that the effective concentration used should be anywhere between 1.5 - 2 $\mu\text{g/ml}$, which is still well within the given EMA guidelines [51].

In this chapter we have introduced a thrombosis-on-a-chip device suitable for hiPSC-EC culturing and whole blood perfusion. Various thrombosis-on-a-chip devices have been introduced before [8,9,11,13–17], recently others have reported on a personalized thrombosis-on-a-chip device using BOECs [12]. However, this is the first report of hiPSC-ECs cultured in microfluidic channels for personalized thrombosis studies. We have determined platelet coverages and platelet aggregate size distributions on untreated and inflamed hiPSC-ECs and compared these to results obtained using HUVECs. Additionally, as a proof-of-principle human whole blood with a clinically relevant concentration of Eptifibatide was perfused through microfluidic channels lined with inflamed hiPSC-ECs which resulted in significantly lower platelet coverages and reduction in adhered platelet aggregate size.

5.5. References

1. WHO Fact Sheet - Cardiovascular diseases Available online: [https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-\(cvds\)](https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds)) (accessed on Jul 15, 2019).
2. Zaragoza, C.; Gomez-Guerrero, C.; Martin-Ventura, J.L.; Blanco-Colio, L.; Lavin, B.; Mallavia, B.; Tarin, C.; Mas, S.; Ortiz, A.; Egido, J. Animal models of cardiovascular diseases. *J. Biomed. Biotechnol.* **2011**, *2011*, 497841.
3. Jirouskova, M.; Shet, A.S.; Johnson, G.J. A guide to murine platelet structure, function, assays, and genetic alterations. *J. Thromb. Haemost.* **2007**, *5*, 661–9.
4. Suo, J.; Ferrara, D.E.; Sorescu, D.; Guldborg, R.E.; Taylor, W.R.; Giddens, D.P.

- Hemodynamic shear stresses in mouse aortas: implications for atherogenesis. *Arterioscler. Thromb. Vasc. Biol.* **2007**, *27*, 346–51.
5. Mak, I.W.; Evaniew, N.; Ghert, M. Lost in translation: animal models and clinical trials in cancer treatment. *Am. J. Transl. Res.* **2014**, *6*, 114–8.
 6. Bhatia, S.N.; Ingber, D.E. Microfluidic organs-on-chips. *Nat. Biotechnol.* **2014**, *32*, 760–772.
 7. Esch, E.W.; Bahinski, A.; Huh, D. Organs-on-chips at the frontiers of drug discovery. *Nat. Rev. Drug Discov.* **2015**, *14*, 248–260.
 8. Westein, E.; van der Meer, A.D.; Kuijpers, M.J.E.; Frimat, J.-P.; van den Berg, A.; Heemskerk, J.W.M. Atherosclerotic geometries exacerbate pathological thrombus formation poststenosis in a von Willebrand factor-dependent manner. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 1357–62.
 9. Costa, P.F.; Albers, H.J.; Linssen, J.E.A.; Middelkamp, H.H.T.; van der Hout, L.; Passier, R.; van den Berg, A.; Malda, J.; van der Meer, A.D. Mimicking arterial thrombosis in a 3D-printed microfluidic in vitro vascular model based on computed tomography angiography data. *Lab Chip* **2017**, *17*.
 10. Mannino, R.G.; Myers, D.R.; Ahn, B.; Wang, Y.; Margo Rollins; Gole, H.; Lin, A.S.; Guldberg, R.E.; Giddens, D.P.; Timmins, L.H.; et al. “Do-it-yourself in vitro vasculature that recapitulates in vivo geometries for investigating endothelial-blood cell interactions”. *Sci. Rep.* **2015**, *5*, 12401.
 11. Menon, N.V.; Tay, H.M.; Wee, S.N.; Li, K.H.H.; Hou, H.W. Micro-engineered perfusable 3D vasculatures for cardiovascular diseases. *Lab Chip* **2017**, *17*, 2960–2968.
 12. Mathur, T.; Singh, K.A.; Rajeeva Pandian, N.K.; Tsai, S.-H.; Hein, T.W.; Gaharwar, A.K.; Flanagan, J.M.; Jain, A. Organ-on-Chips made of Blood: Endothelial Progenitor Cells from Blood reconstitute Vascular Thromboinflammation in Vessel-Chips. *Lab Chip* **2019**.
 13. Barrile, R.; van der Meer, A.D.; Park, H.; Fraser, J.P.; Simic, D.; Teng, F.; Conegliano, D.; Nguyen, J.; Jain, A.; Zhou, M.; et al. Organ-on-Chip Recapitulates Thrombosis Induced by an anti-CD154 Monoclonal Antibody: Translational Potential of Advanced Microengineered Systems. *Clin. Pharmacol. Ther.* **2018**, *104*, 1240–1248.
 14. Zheng, Y.; Chen, J.; Craven, M.; Choi, N.W.; Totorica, S.; Diaz-Santana, A.; Kermani, P.; Hempstead, B.; Fischbach-Teschl, C.; López, J.A.; et al. In vitro microvessels for the study of angiogenesis and thrombosis. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 9342–7.
 15. Jain, A.; Barrile, R.; van der Meer, A.D.; Mammoto, A.; Mammoto, T.; De Ceunynck, K.; Aisiku, O.; Otieno, M.A.; Loudon, C.S.; Hamilton, G.A.; et al. Primary Human Lung Alveolus-on-a-chip Model of Intravascular Thrombosis for Assessment of Therapeutics. *Clin. Pharmacol. Ther.* **2018**, *103*, 332–340.
 16. Zhang, Y.S.; Davoudi, F.; Walch, P.; Manbachi, A.; Luo, X.; Dell’Erba, V.; Miri,

- A.K.; Albadawi, H.; Arneri, A.; Li, X.; et al. Bioprinted thrombosis-on-a-chip. *Lab Chip* **2016**, *16*, 4097–4105.
17. Jain, A.; van der Meer, A.D.; Papa, A.L.; Barrile, R.; Lai, A.; Schlechter, B.L.; Otieno, M.A.; Louden, C.S.; Hamilton, G.A.; Michelson, A.D.; et al. Assessment of whole blood thrombosis in a microfluidic device lined by fixed human endothelium. *Biomed. Microdevices* **2016**, *18*, 1–7.
 18. Cochrane, A.; Albers, H.J.; Passier, R.; Mummery, C.L.; van den Berg, A.; Orlova, V. V.; van der Meer, A.D. Advanced in vitro models of vascular biology: Human induced pluripotent stem cells and organ-on-chip technology. *Adv. Drug Deliv. Rev.* **2018**.
 19. van den Berg, A.; Mummery, C.L.; Passier, R.; van der Meer, A.D. Personalised organs-on-chips: functional testing for precision medicine. *Lab Chip* **2019**, *19*, 198–205.
 20. Takahashi, K.; Tanabe, K.; Ohnuki, M.; Narita, M.; Ichisaka, T.; Tomoda, K.; Yamanaka, S. Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. *Cell* **2007**, *107*, 861–872.
 21. 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. *Nat. Protoc.* **2014**, *9*, 1514–1531.
 22. Orlova, V. V.; Drabsch, Y.; Freund, C.; Petrus-Reurer, S.; Van Den Hil, F.E.; Muenthaion, S.; Ten Dijke, P.; Mummery, C.L. Functionality of endothelial cells and pericytes from human pluripotent stem cells demonstrated in cultured vascular plexus and zebrafish xenografts. *Arterioscler. Thromb. Vasc. Biol.* **2014**, *34*, 177–186.
 23. Choi, K.-D.; Yu, J.; Smuga-Otto, K.; Salvagiotto, G.; Rehrauer, W.; Vodyanik, M.; Thomson, J.; Slukvin, I. Hematopoietic and endothelial differentiation of human induced pluripotent stem cells. *Stem Cells* **2009**, *27*, 559–67.
 24. Adams, W.J.; Zhang, Y.; Cloutier, J.; Kuchimanchi, P.; Newton, G.; Sehrawat, S.; Aird, W.C.; Mayadas, T.N.; Luscinskas, F.W.; García-Cardena, G. Functional vascular endothelium derived from human induced pluripotent stem cells. *Stem cell reports* **2013**, *1*, 105–13.
 25. 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. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 12601–6.
 26. Samuel, R.; Daheron, L.; Liao, S.; Vardam, T.; Kamoun, W.S.; Batista, A.; Buecker, C.; Schäfer, R.; Han, X.; Au, P.; et al. Generation of functionally competent and durable engineered blood vessels from human induced pluripotent stem cells. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 12774–9.
 27. Park, T.S.; Zimmerlin, L.; Zambidis, E.T. Efficient and simultaneous generation of hematopoietic and vascular progenitors from human induced pluripotent stem cells. *Cytom. Part A* **2013**, *83A*, 114–126.

28. Halaidych, O. V.; Freund, C.; van den Hil, F.; Salvatori, D.C.F.; Riminucci, M.; Mummery, C.L.; Orlova, V. V. Inflammatory Responses and Barrier Function of Endothelial Cells Derived from Human Induced Pluripotent Stem Cells. *Stem Cell Reports* **2018**, *10*, 1642–1656.
29. Richardson, M.R.; Yoder, M.C. Endothelial progenitor cells: Quo Vadis? *J. Mol. Cell. Cardiol.* **2011**, *50*, 266–272.
30. Halaidych, O. V.; Cochrane, A.; van den Hil, F.E.; Mummery, C.L.; Orlova, V. V. Quantitative Analysis of Intracellular Ca²⁺ Release and Contraction in hiPSC-Derived Vascular Smooth Muscle Cells. *Stem Cell Reports* **2019**, *12*, 647–656.
31. Nishimura, K.; Sano, M.; Ohtaka, M.; Furuta, B.; Umemura, Y.; Nakajima, Y.; Ikehara, Y.; Kobayashi, T.; Segawa, H.; Takayasu, S.; et al. Development of defective and persistent Sendai virus vector: a unique gene delivery/expression system ideal for cell reprogramming. *J. Biol. Chem.* **2011**, *286*, 4760–71.
32. Zhang, M.; D’Aniello, C.; Verkerk, A.O.; Wrobel, E.; Frank, S.; Ward-van Oostwaard, D.; Piccini, I.; Freund, C.; Rao, J.; Seeböhm, G.; et al. Recessive cardiac phenotypes in induced pluripotent stem cell models of Jervell and Lange-Nielsen syndrome: disease mechanisms and pharmacological rescue. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, E5383–92.
33. Albers, H.J.; Passier, R.; van den Berg, A. van den; van der Meer, A.D. van der Automated Analysis of Platelet Aggregation on Cultured Endothelium in a Microfluidic Chip Perfused with Human Whole Blood. *Micromachines* **2019**, *10*, 781.
34. Colace, T.; Falls, E.; Zheng, X.L.; Diamond, S.L. Analysis of morphology of platelet aggregates formed on collagen under laminar blood flow. *Ann. Biomed. Eng.* **2011**, *39*, 922–929.
35. Hansen, R.R.; Tipnis, A.A.; White-Adams, T.C.; Di Paola, J.A.; Neeves, K.B. Characterization of collagen thin films for von Willebrand factor binding and platelet adhesion. *Langmuir* **2011**, *27*, 13648–13658.
36. Neeves, K.B.; Onasoga, A.A.; Hansen, R.R.; Lilly, J.J.; Venckunaite, D.; Sumner, M.B.; Irish, A.T.; Brodsky, G.; Manco-Johnson, M.J.; Di Paola, J.A. Sources of Variability in Platelet Accumulation on Type 1 Fibrillar Collagen in Microfluidic Flow Assays. *PLoS One* **2013**, *8*.
37. Zack, G.W.; Rogers, E.; Latt, S.A. Automatic Measurement of Sister Chromatid Exchange Frequency. *J. Histochem. Cytochem.* **1977**, *25*, 741–753.
38. Sivarapatna, A.; Ghaedi, M.; Xiao, Y.; Han, E.; Aryal, B.; Zhou, J.; Fernandez-Hernando, C.; Qyang, Y.; Hirschi, K.K.; Niklason, L.E. Engineered Microvasculature in PDMS Networks Using Endothelial Cells Derived from Human Induced Pluripotent Stem Cells. *Cell Transplant.* **2017**, *26*, 1365–1379.
39. Koupenova, M.; Kehrel, B.E.; Corkrey, H.A.; Freedman, J.E. Thrombosis and platelets: an update. *Eur. Heart J.* **2016**, *38*, ehv550.

40. Versaevel, M.; Grevesse, T.; Gabriele, S. Spatial coordination between cell and nuclear shape within micropatterned endothelial cells. *Nat. Commun.* **2012**, *3*, 671.
41. Smith, Q.; Macklin, B.; Chan, X.Y.; Jones, H.; Trempel, M.; Yoder, M.C.; Gerecht, S. Differential HDAC6 Activity Modulates Ciliogenesis and Subsequent Mechanosensing of Endothelial Cells Derived from Pluripotent Stem Cells. *Cell Rep.* **2018**, *24*, 895-908.e6.
42. Charest, J.L.; García, A.J.; King, W.P. Myoblast alignment and differentiation on cell culture substrates with microscale topography and model chemistries. *Biomaterials* **2007**, *28*, 2202–2210.
43. van Hinsbergh, V.W.M. Endothelium—role in regulation of coagulation and inflammation. *Semin. Immunopathol.* **2012**, *34*, 93–106.
44. Wagner, D.D.; Burger, P.C. Platelets in Inflammation and Thrombosis. *Arterioscler. Thromb. Vasc. Biol.* **2003**, *23*, 2131–2137.
45. Bombeli, T.; Schwartz, B.R.; Harlan, J.M. Adhesion of activated platelets to endothelial cells: evidence for a GPIIb/IIIa-dependent bridging mechanism and novel roles for endothelial intercellular adhesion molecule 1 (ICAM-1), α v β 3 integrin, and GPIIb/IIIa. *J. Exp. Med.* **1998**, *187*, 329–39.
46. Wu, K.K.; Thiagarajan, P. ROLE OF ENDOTHELIUM IN THROMBOSIS AND HEMOSTASIS. **1996**.
47. Coenen, D.M.; Mastenbroek, T.G.; Cosemans, J.M.E.M. Platelet interaction with activated endothelium: mechanistic insights from microfluidics. *Blood* **2017**, *130*, 2819–2828.
48. Albers, H.J.; Simão, J.P. da S.; Middelkamp, H.H.T.; Mummery, C.L.; Passier, R.; Berg, A. van den; Orlova, V. V.; Meer, A.D. van der Human induced pluripotent stem cell-derived endothelial cells in thrombosis-on-a-chip devices 2018.
49. B.E., T.; L.K., J.; R.A., H.; D., G.; R.F., P.; D.A., V.; P.R., E.; A.M., L.; M., L.; D.M., J.; et al. Pharmacodynamics and pharmacokinetics of eptifibatide in patients with acute coronary syndromes: Prospective analysis from PURSUIT. *Circulation* **2001**, *104*, 399–405.
50. Gilchrist, I.C.; O'Shea, J.C.; Kosoglou, T.; Jennings, L.K.; Lorenz, T.J.; Kitt, M.M.; Kleiman, N.S.; Talley, D.; Aguirre, F.; Davidson, C.; et al. Pharmacodynamics and pharmacokinetics of higher-dose, double-bolus eptifibatide in percutaneous coronary intervention. *Circulation* **2001**, *104*, 406–411.
51. EMA Integrilin (Eptifibatide) Available online:
https://www.ema.europa.eu/en/documents/product-information/integrilin-epar-product-information_en.pdf (accessed on Aug 16, 2019).
52. FDA Integrilin (Eptifibatide) Available online:
https://www.accessdata.fda.gov/drugsatfda_docs/label/2005/020718s025lbl.pdf (accessed on Aug 16, 2019).

53. van Meer, B.J.; de Vries, H.; Firth, K.S.A.; van Weerd, J.; Tertoolen, L.G.J.; Karperien, H.B.J.; Jonkheijm, P.; Denning, C.; IJzerman, A.P.; Mummery, C.L. Small molecule absorption by PDMS in the context of drug response bioassays. *Biochem. Biophys. Res. Commun.* **2017**, *482*, 323–328.

Chapter 6

Local Stimulation of Endothelium with Inflammatory Factors in 3D-Printed Microfluidic Channels

The study presented in this chapter is based on the work done in collaboration with Simone ten Den, Kelly Klaassen and Meike Große-Elshoff during their internships or graduation projects for obtaining their Bachelor of Applied Sciences degree in Biology & Medical Laboratory Research.

Abstract

Vascular inflammation *in vivo* often occurs at specific locations with aberrant flow, *e.g.* stenotic or bifurcating geometries. This study introduces a thrombosis-on-a-chip model aimed to locally activate endothelium for thrombosis studies. A 3D-printed mold was used to pattern two perpendicular channels with a circular cross-section using standard PDMS soft lithography. A crescent shaped semi-permeable collagen-I barrier was patterned to provide a matrix for endothelial cells to adhere to while still allowing diffusion of diluted species. Inflammatory cytokines were introduced in the secondary channel resulting in activated endothelium and platelet aggregation during human whole blood perfusion in the main endothelialized channel.

6.1. Introduction

Cardiovascular diseases (CVDs) are currently the number one cause of death worldwide, mostly attributable to acute myocardial infarction or cerebrovascular ischemic events [1]. These events are mostly caused by thrombosis, the aberrant formation of intravascular blood clots. To understand, prevent and treat arterial thrombosis, various model systems are used in biomedical research. Animal models, *e.g.* murine models, are popular in thrombosis research due to their multifactorial nature, integrating blood-borne as well as vascular factors [2]. Unfortunately, when investigating thrombosis, key parameters such as fluid dynamics, platelet biology, *etc.* in murine models differ significantly from the human biology [3–5]. Porcine models offer realistic alternatives, but are expensive and are also not fully representative of human thrombosis [2]. Therefore, organ-on-a-chip models are of interest. These microfluidic models combine the flexibility of *in vitro* models with the realism and complexity of animal models.

Various *in vitro* models for vasculature and thrombosis studies have been reported, ranging from flow chambers with exposed collagen regions mimicking vascular damage [6–10], to models incorporating a full endothelial monolayer [11–20]. Microfluidic models use various techniques to produce channels with rectangular [12,14,15,17–19] or round cross-sections [11,13,16,20] and allow stenotic [11,12,19,20], bifurcating [13,19,20] or even network geometries [16].

In microfluidic endothelialized models, or ‘thrombosis-on-a-chip’ models, thrombosis is often induced by introducing inflammatory cytokines to activate the endothelial monolayer [12,14,17–19]. This results in a fully inflamed endothelial monolayer that in turn activates platelets and leads to aggregation and thrombosis. However, *in vivo* vascular inflammation does not develop randomly but occurs at specific sites in arteries, often found in the vicinity of bifurcations or areas with aberrant flow, *i.e.* recirculation [21]. Others have investigated the effect of various types of flow on endothelial cells cultured in microfluidic channels by exposing the endothelial cells to flows ranging from static conditions to chaotic flow profiles [22]. In addition to these local flow patterns, it would be useful to be able to induce local inflammation by spatially confined treatment with inflammatory cytokines.

A microfluidic device with two perpendicular channels separated by a semi-permeable barrier could be used to locally induce inflammation. Others have reported on fabricating devices fitting this description using standard wafer based soft lithography techniques [8–10,23,24]. However, these devices have square or rectangular cross-section channels which inevitably create low flow zones in the corners recreating a venous-like zone in the corners and arterial-like zone in the middle of the channels [25,26]. This issue can be partially addressed by omitting the data generated in low flow zones. Alternatively, the problem can be solved by fabricating circular cross-section channels, which have uniform wall shear rate profiles [11].

In this study, we present a 3D-printed microfluidic model used for thrombo-inflammation studies using two perpendicular round channels to locally induce inflammation. An endothelialized main channel is separated from a second channel by a semi-permeable collagen barrier. By locally allowing diffusion of an inflammatory cytokine from the secondary into the main channel, endothelium could be inflamed. This chapter discusses the fabrication process and biocompatibility of the 3D-printed molds. Furthermore, a proof-of-concept study is presented where human umbilical vein endothelial cells (HUVECs) were cultured in the device. After activation of the HUVECs, human whole blood was perfused through the endothelialized channel while monitoring platelet aggregation.

6.2. Materials & Methods

6.2.1. 3D-Printing

3D-printing was conducted with a Form 2 (Formlabs) using dental SG resin. Using proprietary software (PreForm) the mold designs were positioned on the build platform plane, with an offset of 45° respective to the two planes perpendicular to the build platform to prevent overhang. Reinforcements in the form of auto-generated support structures were added in PreForm with a density of 1 and point size of 0.9 mm. After printing, the structures were removed from the build platform, washed twice in isopropyl alcohol (IPA) for 10 minutes while taking additional care to flush the cavities to prevent any residual resin from cross-linking. The supported molds were dried using a nitrogen gun and finished in the FormCure (Formlabs), an automatic post-curing chamber, for 120 minutes

at 60 °C. Parts were autoclaved at 121 °C for 20 minutes (Varioklav) and finally exposed to 1000W UV light (peak at $\lambda = 360$ nm) for 60 seconds.

6.2.2. Chip Fabrication

A steel wire ($\varnothing = 1$ mm) and an acupuncture needle ($\varnothing = 300$ μ m) were inserted into the molds. The 3D-printed mold was attached to a glass microscopy slide using UV-curable glue (Norland Optical Adhesive 81) and cross-linked using a custom UV-gun with a power of 400 mW/cm² for 10 seconds.

Polydimethylsiloxane (PDMS) pre-polymer and curing agent were mixed at a 10:1 ratio, degassed and divided over multiple molds. Molds and PDMS were quickly degassed to remove any bubbles trapped during PDMS casting. The PDMS was cross-linked for 75 minutes at 60 °C, after which the PDMS chips were removed from the molds and glass slides. The PDMS chips with the wires were placed back into an oven for an additional 120 minutes at 60 °C. This resulted in a fully cross-linked PDMS device with an opening between the main channel (300 μ m $\times$ 12 mm, diameter $\times$ length) and the secondary channel (1 mm $\times$ 8 mm, diameter $\times$ length).

The PDMS chip was exposed to plasma, generated using air at a pressure of 500 mTorr using a power of 50W at a frequency of 5 kHz (Femto Science, CUTE). The upper secondary channel was coated with a 3% (v/v) (3-Aminopropyl)triethoxysilane (APTES, Sigma Aldrich) in MilliQ and a 10% (v/v) Glutaraldehyde (Sigma Aldrich, GA) in phosphate-buffered saline (PBS) consecutively. Each solution was allowed to coat the secondary channel for 30 minutes and was removed with an ethanol (EtOH) wash followed by a nitrogen purge.

A 6 mg/ml collagen-I (Rat-tail high concentration, Corning) gel was made in a chilled Eppendorf tube using sterile de-ionized water, 10x PBS (Sigma Aldrich) and 1M NaOH (Sigma Aldrich). A collagen barrier was patterned by introducing the chilled collagen-I into the secondary channel followed by a wire with a diameter of 780 μ m and an acupuncture needle in the main channel. By incubating the chip upside-down for 30 minutes at 37 °C, a crescent shape collagen-I barrier was formed. After 30 minutes the wire and acupuncture needle are removed and the lower main channel was coated with 0.1 mg/ml collagen-I (Rat-tail high concentration, Corning) by incubation at 37 °C for 30 minutes.

6.2.3. Cell Seeding

The chip was thoroughly flushed with endothelial cell growth medium (ECGM, Cell Applications) and incubated for 30 minutes at 37 °C. An ~80% confluent T75 with human umbilical vein endothelial cells (HUVECs) was trypsinized and the cell suspension centrifuged for 5 minutes at 390g. The supernatant was aspirated and pellet re-suspended to create a cell suspension with a final concentration of 10×10^6 cells/ml. Using a pipette, 5 μ l cell suspension was introduced in the main channel and incubated for 30 minutes. During incubation a second ~80% confluent T75 was processed for the second seeding covering the top of the microfluidic channel by incubating topside-down. Microfluidic chips were placed into a 12 well plate, with ECGM to prevent the channels from drying during incubation. Endothelial cells were incubated for 48h at 37 °C to reach confluency, while replacing ECGM twice per 24h.

Inflammation was instigated by introducing ECGM with 50 ng/ml tumor necrosis factor- α (TNF- α , Sigma Aldrich) to the secondary channel and incubating for 4h at 37 °C.

Biocompatibility studies were conducted in 96 well-plates, where HUVECs were seeded at 1.2×10^6 cells/ml and cultured for 24h at 37 °C. After the cells formed a confluent monolayer, one of four conditions was applied for 24h in each of the wells: ECGM exposed (for 24h) to a 3D-printed construct autoclaved once (1) or twice (2), untreated ECGM (3) and ECGM with 10% EtOH (4). To assess the effect of each condition, a Live/Dead assay for mammalian cells in conjunction with a NucBlue nuclear probe kit was used per manufacturers' instructions.

Fluorescence microscopy images of endothelial cell morphology were made after fixating the monolayer with 4% formaldehyde (v/v, Sigma Aldrich) and permeabilization with 0.3% Triton X-100 (v/v, Sigma Aldrich), both at room temperature for 30 minutes. NucBlue and ActinGreen probe kits were used to stain nuclei and F-actin and were used per manufacturer's instruction. Fluorescence microscopy images of biocompatibility and morphology of endothelial cells were made using an EVOS Imaging System (Invitrogen) using 4 $\times$ and 10 $\times$ objectives.

6.2.4. Human Whole Blood Perfusion

Human whole blood was provided by the Experimental Centre for Technical Medicine, which is part of the Technical Medical Centre of the University of Twente. The blood was used within 4h of being drawn (Greiner Bio-One, Vacuette 3.2% sodium citrate). To allow coagulation to occur, the blood was re-calcified using a 1:10 (v/v) of re-calcification buffer (HEPES, Gibco, 63.2 mM CaCl₂, Sigma Aldrich, 31.6 mM MgCl₂, Ambion) to whole blood.

Re-calcified citrated human whole blood was perfused at a shear rate of 160 s⁻¹ for a period of 9 minutes. To visualize platelet aggregation 1% (v/v) CD41-PE (ThermoFisher) was added to the whole blood 10 minutes prior to whole blood perfusion. Platelet aggregation was monitored on a Leica DM IRM using a 10× objective.

6.3. Results & Discussion

Fabrication of the microfluidic devices consisted of mold fabrication and PDMS soft lithography resulting in a microfluidic device with two circular and perpendicular channels. A collagen barrier between the two channels allowed endothelial cells to be cultured in the main channel and inflammatory cytokines, to be introduced through the secondary channel, for local inflammation.

This section explains the design and fabrication process of these microfluidic devices. Furthermore, biocompatibility of the Dental SG resin was investigated. Endothelial cells were cultured in the main microfluidic channel and whole blood perfusion was conducted.

6.3.1. Mold and Chip Fabrication

6.3.1.1. PDMS Chip Fabrication

A rectangular mold was designed in SolidWorks (Dassault Systèmes) with four cylindrical holes to position a metal wire and needle for PDMS soft lithography (Figure 1A). The holes guiding and spacing the metal wire and needle were designed to overlap by 30 µm, to put tension on the metal wire and needle. This prevented PDMS from seeping in between the metal wire and needle during soft lithography and ensured an open connection to occur. The rectangular molds with inserted metal wires and needles were glued to microscopy slides and

PDMS was cast, degassed and cross-linked creating a negative of the molds (Figure 1B-C).

The resulting PDMS chips had two perpendicular channels connected by a small opening. In the secondary channel a collagen barrier was patterned to keep endothelial cells from moving into the secondary channel while simultaneously allowing inflammatory cytokines to pass the barrier (Figure 1D).

Figure 1. Microfluidic chip fabrication. (A) Render of a 3D-printed mold with a steel wire and needle for channel patterning. (B) Render of PDMS chip produced using the 3D-printed mold. (C) Fabricated 3D-printed chip. (D) Schematic cross-section of the two microfluidic channels including a crescent shaped collagen barrier. Scale bar, 8 mm.

The 3D-printed mold and the guiding holes determined the spacing between the wire and needle. Therefore, the diameter of guide holes of ten molds was investigated by printing a guide hole with an intended diameter of 650 μm followed by a measurement of the actual printed diameter. This resulted in a mean diameter of $618 \pm 10.8 \mu\text{m}$. Despite this deviation, the 30 μm overlap between the wire and needle was enough to prevent the main and secondary channel from not being connected.

Besides the introduced molds, that use a needle and wire for main and secondary channel patterning, molds with a 3D-printed arterial structure were

produced using the Form 2 3D-printer. Effectively reproducing the molds introduced in Chapter 3, with the addition of a secondary channel (Supplementary Figure D-1). The 3D-printed artery-like features suffered from 3D-printing artefacts in the form of irregularities and surface roughness resulting in a structure deviating from the designed shapes creating unpredictable shear rate profiles (Supplementary Figure D-2).

6.3.1.2. Collagen Barrier Functionality

The 3D-printed mold used for soft lithography of PDMS creates two perpendicular channels with a small opening between the two channels. To be able to locally induce inflammation or affect the endothelial cells, a barrier between the two channels needs to be established. This barrier should allow to transport of diluted species while still providing a matrix to which the endothelial cells can attach.

To establish this barrier, a high concentration of collagen-I (6 mg/ml) was introduced in the large secondary channel. Before gelation of the collagen, a metal wire with a slightly smaller diameter than the large channel ($\varnothing = 780 \mu\text{m}$) was pushed into the channel. By incubating the chip upside down, a crescent-shaped collagen barrier was formed (Figure 1D).

As a proof-of-principle the barrier functionality was tested using fluorescently labeled beads ($\varnothing = 6 \mu\text{m}$) and fluorescein representing endothelial cells in suspension and inflammatory cytokines or drugs respectively. Fluorescein should be able to pass through the collagen layer while the beads should be inhibited from being transported from one channel to the perpendicular channel. Ideally the beads would have been introduced in the main channel, replicating the endothelial cell seeding. Nevertheless, this result shows that the collagen layer acts as a barrier for spherical objects with a smaller diameter than endothelial cells in suspension while small molecules can still pass.

Figure 2. Collagen barrier functionality. (A) Beads with a diameter of 6 μm representing seeded endothelial cells are retained in one channel. (B) Fluorescein is introduced in the upper channel. After 4h a detectable concentration of fluorescein is measured in the main channel. Scale bar, 1 mm.

The main and secondary channels are designed to allow diffusion of inflammatory cytokines through the collagen barrier while inhibiting transport of endothelial cells in suspension. Figure 2B shows a proof of principle using fluorescein, which has a diffusion coefficient of one magnitude higher than $\text{TNF-}\alpha$. Figure 2B shows the collagen barrier does allow transport of diluted species, but the speed at which fluorescein diffuses through the barrier is not representative for $\text{TNF-}\alpha$.

6.3.2. Incorporation of Endothelial Cells

6.3.2.1. Biocompatibility

Dental SG resin was used to print molds for PDMS soft lithography. The manufacturer (Formlabs) designed the resin to be biocompatible for uses as surgical guides in dental applications [27]. To test whether the used post-curing steps were sufficient to ensure biocompatibility a Live/Dead assay was conducted.

The 3D-printed mold was washed in IPA twice, post-cured in the FormCure, autoclaved and exposed to a high intensity UV treatment for one minute. Microfluidic molds were sterilized in 70% EtOH, thoroughly dried and placed in ECGM for 24h. The ECGM was used to indirectly test the biocompatibility of the 3D-printed molds by checking whether any leached out trace elements might affect the endothelial cells.

A confluent layer of HUVECs cultured in a well plate was incubated with ECGM exposed to a 3D-printed mold autoclaved once (Figure 3A) or twice (Figure 3B) and to a negative (Figure 3C, ECGM) and positive control (Figure 3D, ECGM + 10% EtOH).

Figure 3. Biocompatibility of molds fabricated with dental resin in well plates. (A-B) HUVECs cultured in ECGM exposed to autoclaved mold for 24h where the mold was autoclaved once (A) or twice (B). (C-D) Negative control of HUVECs cultured in ECGM (C) and positive control of HUVECs cultured in ECGM + 10% EtOH (D). Nuclei in blue, live cells in green and dead cells in red. Scale bar, 250 μm .

Microscopy images of HUVECs incubated for 24h in ECGM exposed to a 3D-printed mold, regardless of the number of times autoclaved, resemble the images of HUVECs in the negative control. The positive control paints a different picture, with no live cells and a few attached dead cells. Based on these results we can conclude that, in combination with the earlier mentioned post-curing steps, a single sterilization in an autoclave is sufficient to ensure biocompatibility.

6.3.2.2. Endothelial Cells Form Confluent Monolayer

Endothelial cells were introduced in the main channel at a seeding density of 10×10^6 cells/ml and were allowed to reach confluency in 48h. The collagen barrier

keeps the endothelial cells from being seeded in the large secondary channel. To check whether the endothelial cells form a confluent monolayer, the cells were fixated, permeated and stained (nuclei and F-actin). Figure 4 shows a low and high magnification image of endothelial coverage in the main channel, where figure 4B shows a section equal to 1/8th of the full channel and figure 4C a higher magnification displaying HUVEC morphology.

Figure 4. (A) Render of PDMS chip indicating the main channel. (B-C) Endothelial cells cultured in microfluidic channel forming a confluent monolayer. Nuclei in blue, F-actin in green. Scale bars, 300 μm .

The endothelial cells were able to form a confluent monolayer after 48h of culture in the main channel. Figure 4B shows HUVECs with a stretched cobblestone-like morphology and even F-actin distribution. However, HUVECs with a less stretched morphology are also to be found which could indicate overseeding.

6.3.3. Whole Blood Perfusion

6.3.3.1. Activated Endothelium

After allowing the HUVECs to reach confluency in the main channel, an inflammatory cytokine TNF- α was introduced in the secondary channel and incubated for a period of 4h. The diffusion coefficient of TNF- α is $3.52 \times 10^{-7} \text{ cm}^2/\text{s}$ [28] and can be used to calculate the mean traveled diffusion radius, assuming 3D diffusion, equal diffusion coefficient in the collagen barrier and a steady state, by using $\bar{x} = \sqrt{6Dt}$, where $\bar{x}$, mean traveled distance (m), D, diffusion coefficient (m^2/s) and t, elapsed time (s). This resulted in a mean diffusion radius of $\bar{x} = 1.74$

mm, thus approximately a third of the main channel is affected by $\text{TNF-}\alpha$. This affected area is likely to be an overestimation, since the diffusion coefficient in the collagen barrier will be lower, but this calculation does indicate that the chosen exposure time should induce a local inflammation.

Inflamed endothelial cells express various surface markers and release pro-inflammatory factors which allow platelet aggregation [29–34]. To test whether the inflamed HUVECs cause platelet aggregation, re-calcified citrated human whole blood was perfused at a shear rate of 160 s^{-1} for a period of approximately 8 minutes. Platelets were labeled using CD41-PE, and fluorescence microscopy images were recorded right next to the intersection of the main and secondary channel. Three representative fluorescence microscopy images of platelet aggregation after 180, 360 and 520 seconds are given in Figure 5A-C, which show an increase in platelet coverage over time.

Figure 5. Microfluidic device lined with inflamed HUVECs perfused with re-calcified citrated human whole blood at a shear rate of 160 s^{-1} . Platelets were stained using CD41-

PE (grey) and shown at various time points: 180 s (A), 360 s (B) and 520 s (C). Platelet adhesion was monitored in a region in close proximity to the collagen barrier. Scale bar, 150 μm .

The first three minutes of data are not shown in Figure 5, due to misalignment issues after attachment of tubing. Three minutes after the start of whole blood perfusion a region of interest close to the intersection of the main and secondary channel was found and data was acquired.

Results from one microfluidic device are presented in Figure 5. In future studies, a control experiment with inactivated endothelium could be used to determine baseline platelet adhesion.

6.4. Conclusions

In this chapter, a fabrication technique for creating a microfluidic device with two perpendicular channels with circular cross-section was introduced and characterized. A biocompatibility study of the used dental SG resin was conducted showing that the chosen post-curing steps are sufficient to ensure a high degree of biocompatibility. Furthermore, as a proof-of-principle, HUVECs were cultured for 48h in microfluidic channels. After the endothelial cells formed a confluent monolayer, inflammation was induced from the secondary channel. The pro-thrombotic properties of the inflamed endothelium were investigated by perfusion of re-calcified citrated human whole blood where an increase in platelet coverage over time was observed.

6.5. Recommendations

Various chip holders have been investigated (Supplementary Figure D-1) but all designs were prone to leakage. In the microfluidic device the main channel is positioned quite close to the bottom of the device to ensure compatibility with confocal microscopy. When placing a PDMS chip into a chipholder, the PDMS acts as a gasket, but because the channel is positioned in close proximity to the chipholder edges this often results in leakages. By increasing this distance in both the microfluidic chip and the chipholder, fewer leakages should occur. To still be able to connect the chip to a syringe pump for whole blood perfusion, 30 gauge needles were inserted into the main channel. To provide a secure connection a

significant portion of the needle had to be inserted into the channel, which reduced the effective length of the main channel and obstructed fluorescence microscopy close to the inlets and outlets. The use of a chipholder should result in lower hydraulic resistance which allows whole blood perfusion at higher shear rates. Implementing a chip holder also allows the inlets and outlets to be monitored during whole blood perfusion. Furthermore, it makes performing basic operations with the device, e.g. replacing medium and whole blood perfusion, significantly easier.

A second recommendation is to repeat the whole blood perfusion experiments using various exposure times to the inflammatory cytokine including a control condition.

Besides inducing local inflammation in endothelial cells, the intersection of the two channels can be used to mimic acute vascular damage. By creating a confluent monolayer of HUVECs and actively pushing a pro-thrombotic material like collagen from the secondary into the main channel, a vascular fissure can be mimicked. Others have reported on similar two channel devices with exposed collagen before [8], but not using channels with a circular cross-section.

Currently the 3D-printed construct is used for PDMS molding. Alternatively, different materials like the hydrogel Gelatin methacryloyl (GelMA) could be used to fabricate the two channels. By creating a well-defined distance between the main and secondary channel, diffusion through the hydrogel could result in local inflammation.

6.6. References

1. WHO Fact Sheet - Cardiovascular diseases Available online: [https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-\(cvds\)](https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds)) (accessed on Jul 15, 2019).
2. Zaragoza, C.; Gomez-Guerrero, C.; Martin-Ventura, J.L.; Blanco-Colio, L.; Lavin, B.; Mallavia, B.; Tarin, C.; Mas, S.; Ortiz, A.; Egido, J. Animal models of cardiovascular diseases. *J. Biomed. Biotechnol.* **2011**, *2011*, 497841.
3. Jirouskova, M.; Shet, A.S.; Johnson, G.J. A guide to murine platelet structure, function, assays, and genetic alterations. *J. Thromb. Haemost.* **2007**, *5*, 661–9.
4. Suo, J.; Ferrara, D.E.; Sorescu, D.; Guldberg, R.E.; Taylor, W.R.; Giddens, D.P. Hemodynamic shear stresses in mouse aortas: implications for atherogenesis. *Arterioscler. Thromb. Vasc. Biol.* **2007**, *27*, 346–51.
5. Lemini, C.; Jaimez, R.; Franco, Y. Gender and inter-species influence on

- coagulation tests of rats and mice. *Thromb. Res.* **2007**, *120*, 415–419.
6. Hansen, R.R.; Tipnis, A.A.; White-Adams, T.C.; Di Paola, J.A.; Neeves, K.B. Characterization of collagen thin films for von Willebrand factor binding and platelet adhesion. *Langmuir* **2011**, *27*, 13648–13658.
7. Neeves, K.B.; Onasoga, A.A.; Hansen, R.R.; Lilly, J.J.; Venckunaite, D.; Sumner, M.B.; Irish, A.T.; Brodsky, G.; Manco-Johnson, M.J.; Di Paola, J.A. Sources of Variability in Platelet Accumulation on Type 1 Fibrillar Collagen in Microfluidic Flow Assays. *PLoS One* **2013**, *8*.
8. Muthard, R.W.; Diamond, S.L. Side view thrombosis microfluidic device with controllable wall shear rate and transthrumbus pressure gradient. *Lab Chip* **2013**, *13*, 1883–91.
9. Muthard, R.W.; Welsh, J.D.; Brass, L.F.; Diamond, S.L. Fibrin, γ' -Fibrinogen, and Transclot Pressure Gradient Control Hemostatic Clot Growth During Human Blood Flow Over a Collagen/Tissue Factor Wound. *Arterioscler. Thromb. Vasc. Biol.* **2015**, *35*, 645–654.
10. Muthard, R.W.; Diamond, S.L. Blood Clots Are Rapidly Assembled Hemodynamic Sensors. *Arterioscler. Thromb. Vasc. Biol.* **2012**, *32*, 2938–2945.
11. Costa, P.F.; Albers, H.J.; Linssen, J.E.A.; Middelkamp, H.H.T.; van der Hout, L.; Passier, R.; van den Berg, A.; Malda, J.; van der Meer, A.D. Mimicking arterial thrombosis in a 3D-printed microfluidic in vitro vascular model based on computed tomography angiography data. *Lab Chip* **2017**, *17*.
12. Westein, E.; van der Meer, A.D.; Kuijpers, M.J.E.; Frimat, J.-P.; van den Berg, A.; Heemskerk, J.W.M. Atherosclerotic geometries exacerbate pathological thrombus formation poststenosis in a von Willebrand factor-dependent manner. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 1357–62.
13. Zhang, Y.S.; Davoudi, F.; Walch, P.; Manbachi, A.; Luo, X.; Dell’Erba, V.; Miri, A.K.; Albadawi, H.; Arneri, A.; Li, X.; et al. Bioprinted thrombosis-on-a-chip. *Lab Chip* **2016**, *16*, 4097–4105.
14. Jain, A.; van der Meer, A.D.; Papa, A.-L.; Barrile, R.; Lai, A.; Schlechter, B.L.; Otieno, M.A.; Loudon, C.S.; Hamilton, G.A.; Michelson, A.D.; et al. Assessment of whole blood thrombosis in a microfluidic device lined by fixed human endothelium. *Biomed. Microdevices* **2016**, *18*, 73.
15. Jain, A.; Barrile, R.; van der Meer, A.D.; Mammoto, A.; Mammoto, T.; De Ceunynck, K.; Aisiku, O.; Otieno, M.A.; Loudon, C.S.; Hamilton, G.A.; et al. Primary Human Lung Alveolus-on-a-chip Model of Intravascular Thrombosis for Assessment of Therapeutics. *Clin. Pharmacol. Ther.* **2018**, *103*, 332–340.
16. Zheng, Y.; Chen, J.; Craven, M.; Choi, N.W.; Totorica, S.; Diaz-Santana, A.; Kermani, P.; Hempstead, B.; Fischbach-Teschl, C.; López, J.A.; et al. In vitro microvessels for the study of angiogenesis and thrombosis. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 9342–7.
17. Barrile, R.; van der Meer, A.D.; Park, H.; Fraser, J.P.; Simic, D.; Teng, F.;

- Conegliano, D.; Nguyen, J.; Jain, A.; Zhou, M.; et al. Organ-on-Chip Recapitulates Thrombosis Induced by an anti-CD154 Monoclonal Antibody: Translational Potential of Advanced Microengineered Systems. *Clin. Pharmacol. Ther.* **2018**, *104*, 1240–1248.
18. Mathur, T.; Singh, K.A.; R. Pandian, N.K.; Tsai, S.-H.; Hein, T.W.; Gaharwar, A.K.; Flanagan, J.M.; Jain, A. Organ-on-chips made of blood: endothelial progenitor cells from blood reconstitute vascular thromboinflammation in vessel-chips. *Lab Chip* **2019**, *19*, 2500–2511.
19. Menon, N.V.; Tay, H.M.; Wee, S.N.; Li, K.H.H.; Hou, H.W. Micro-engineered perfusable 3D vasculatures for cardiovascular diseases. *Lab Chip* **2017**, *17*, 2960–2968.
20. Mannino, R.G.; Myers, D.R.; Ahn, B.; Wang, Y.; Margo Rollins; Gole, H.; Lin, A.S.; Guldborg, R.E.; Giddens, D.P.; Timmins, L.H.; et al. “Do-it-yourself in vitro vasculature that recapitulates in vivo geometries for investigating endothelial-blood cell interactions”. *Sci. Rep.* **2015**, *5*, 12401.
21. Gimbrone, M.A.; García-Cardena, G. Endothelial Cell Dysfunction and the Pathobiology of Atherosclerosis. *Circ. Res.* **2016**, *118*, 620–636.
22. Varma, S.; Garcia-cardena, G.; Voldman, J. Unraveling Endothelial Cell Phenotypic Regulation By Spatial Hemodynamic Flows With Microfluidics. *Proc MicroTAS 2018* **2018**, 109–112.
23. Griep, L.M.; Wolbers, F.; De Wagenaar, B.; Ter Braak, P.M.; Weksler, B.B.; Romero, I.A.; Couraud, P.O.; Vermes, I.; Van Der Meer, A.D.; Van Den Berg, A. BBB on CHIP: Microfluidic platform to mechanically and biochemically modulate blood-brain barrier function. *Biomed. Microdevices* **2013**, *15*, 145–150.
24. van der Helm, M.W.; Odijk, M.; Frimat, J.P.; van der Meer, A.D.; Eijkel, J.C.T.; van den Berg, A.; Segerink, L.I. Direct quantification of transendothelial electrical resistance in organs-on-chips. *Biosens. Bioelectron.* **2016**, *85*, 924–929.
25. van der Helm, M.W.; van der Meer, A.D.; Eijkel, J.C.T.; van den Berg, A.; Segerink, L.I. Microfluidic organ-on-chip technology for blood-brain barrier research. *Tissue Barriers* **2016**, *4*.
26. Albers, H.J.; Passier, R.; van den Berg, A. van den; van der Meer, A.D. van der Automated Analysis of Platelet Aggregation on Cultured Endothelium in a Microfluidic Chip Perfused with Human Whole Blood. *Micromachines* **2019**, *10*, 781.
27. Formlabs Dental SG Resin Available online: https://support.formlabs.com/s/article/Using-Dental-SG-Resin?language=en_US (accessed on Oct 23, 2019).
28. Harm, S.; Schildböck, C.; Hartmann, J. Cytokine Removal in Extracorporeal Blood Purification: An in vitro Study. *Blood Purif.* **2019**, 1–11.
29. Koupenova, M.; Kehrel, B.E.; Corkrey, H.A.; Freedman, J.E. Thrombosis and platelets: an update. *Eur. Heart J.* **2016**, *38*, ehv550.
30. van Hinsbergh, V.W.M. Endothelium —role in regulation of coagulation and

- inflammation. *Semin. Immunopathol.* **2012**, 34, 93–106.
31. Bombeli, T.; Schwartz, B.R.; Harlan, J.M. Adhesion of activated platelets to endothelial cells: evidence for a GPIIb/IIIa-dependent bridging mechanism and novel roles for endothelial intercellular adhesion molecule 1 (ICAM-1), α v β 3 integrin, and GPIb α . *J. Exp. Med.* **1998**, 187, 329–39.
 32. Coenen, D.M.; Mastenbroek, T.G.; Cosemans, J.M.E.M. Platelet interaction with activated endothelium: mechanistic insights from microfluidics. *Blood* **2017**, 130, 2819–2828.
 33. Wagner, D.D.; Burger, P.C. Platelets in Inflammation and Thrombosis. *Arterioscler. Thromb. Vasc. Biol.* **2003**, 23, 2131–2137.
 34. Wu, K.K.; Thiagarajan, P. ROLE OF ENDOTHELIUM IN THROMBOSIS AND HEMOSTASIS. **1996**.

Chapter 7

Summary & Outlook

7.1. Summary

Cardiovascular diseases (CVDs) are the number one cause of death, often caused by arterial thrombosis, *i.e.* the formation of a blood clot inside of a blood vessel. Great efforts are made to develop therapeutics to prevent or cure CVDs in general, and arterial thrombosis in particular. Unfortunately, current pre-clinical studies have poor predictive power and thus there is a need for realistic models with improved predictive power for human efficacy and toxicity. In **Chapter I** an overview of vasculature- and thrombosis-on-a-chip models was given. The chapter concluded that there is a need for thrombosis-on-a-chip devices with disease-specific geometries, person-specific endothelial cells, automated data analysis methodology and the possibility to locally activate endothelial cells. These possible improvements were discussed in the experimental **Chapters III – VI**. However, first an overview of the use of human induced pluripotent stem cells (hiPSCs) for vascular models and their use in organ-on-a-chip devices was given in **Chapter II**. Various protocols to differentiate hiPSCs to endothelial cells, pericytes and vascular smooth muscle cells have been introduced, which are the basic building blocks of vasculature. These hiPSC-derived cells could be used to mimic vascular dysfunction. Introducing these hiPSC-derived cells into organ-on-a-chip devices could open up opportunities to move towards a personalized approach. In addition to using person-specific vascular cells to create personalized devices, also the geometry of the microfluidic device is of importance. In **Chapter III**, human computed tomography angiography data was used to model a 3D-printed mould with and without a stenotic geometry. These moulds were used to fabricate polydimethylsiloxane (PDMS) devices that were lined with human umbilical vein endothelial cells (HUVECs) and perfused with re-calcified citrated human whole blood. Healthy geometries did not show any sign of thrombosis, while the stenotic geometries did show thrombosis at the apex and distal of the stenosis. In **Chapter IV** an automated methodology was introduced to process end-point fluorescence microscopy images of whole blood perfusion in microfluidic channels lined with untreated or inflamed endothelium. The methodology determines platelet coverage and platelet aggregate size distributions and was compared to manually identifying single platelets and platelet aggregates. The introduced methodology shows a high degree of sensitivity, specificity and accuracy. This methodology was used in

Chapter V to quantitatively compare platelet aggregation on untreated and inflamed HUVECs and human induced pluripotent stem cell-derived endothelial cells (hiPSC-ECs) cultured in microfluidic channels after whole blood perfusion. No significant differences between HUVECs and hiPSC-ECs were found. As a proof-of-principle, the anti-platelet drug Eptifibatide was added to whole blood perfused through a microfluidic device lined with inflamed hiPSC-ECs resulting in significantly lower platelet aggregation. **Chapter VI** discussed the fabrication of a 3D-printed microfluidic device for the local stimulation of endothelial cells in a circular cross-section channel. The biocompatibility of the used 3D-printed resin was tested and a proof-of-principle whole blood perfusion through a channel lined with HUVECs was conducted. After the summary and outlook in **Chapter VII** several **Appendices** discuss preliminary results of the application of thrombosis-on-a-chip models, obtained in collaboration with various university medical centers.

7.2. Outlook and Future Work

This section will elaborate on improvements introduced in previous chapters and their effect on the realism and predictive value of the models. Furthermore, preliminary results obtained in collaboration with various university medical centers utilizing the introduced thrombosis-on-a-chip models and their future perspectives are briefly discussed.

7.2.1. Human Induced Pluripotent Stem Cell-Derived Cells

In the studies presented in this thesis primary human endothelium and hiPSC-ECs were cultured in thrombosis-on-a-chip devices. The implementation of hiPSC-ECs in thrombosis-on-a-chip devices brings us one step closer to a personalized medicine approach [1].

The presented study in Chapter V has been conducted with hiPSC-ECs generated using one healthy control donor. Since the hiPSC-ECs are person-specific, individual genetic predispositions should result in hiPSC-derived cells also being more susceptible towards certain diseases. Therefore, it would be of great interest to generate patient-specific endothelial cells and implement these in organ-on-chip devices. Implementing patient-specific hiPSC-ECs would allow us to study diseases, *e.g.* von Willebrand disease and pulmonary arterial

hypertension [2] in a realistic setting. Furthermore, hiPSCs from patients can be genetically modified using techniques like CRISPR/Cas9 to investigate and understand disease mechanics and pathways.

Organ-on-a-chip technology has enabled us to perfuse microfluidic channels with whole blood to study thrombosis. It has been reported that mechanical stimuli like shear can induce a more mature and arterial phenotype in endothelial cells [3,4]. Therefore, it would be interesting investigate the role of prolonged shear rate exposure on hiPSC-ECs prior to whole blood perfusion.

It has been shown that brain perivascular cells influence inflammatory response in a co-culture with endothelial cells [5]. Recently others have reported on differentiating vascular smooth muscle cells from hiPSCs [6]. An exciting next step would be to combine hiPSC-derived endothelium and smooth muscle cells, from one donor, to investigate inflammatory and pro-thrombotic properties.

7.2.2. Microfluidic Channel Architectures

Throughout the thesis various microfluidic channel geometries were introduced, ranging from simple yet reproducible rectangular channel cross-sections to complex circular channel cross-sections. Chapter III introduced a microfluidic chip based on human computed tomography angiography (CTA) data. A 3D-model of a coronary artery was used and modified to display various degrees of occlusion. Alternatively, scans of patients suffering from occluded arteries could be used to investigate the fluid dynamics and pro-thrombotic properties of these stenotic geometries. Furthermore, not only occluding geometries but also aneurysms could be investigated. Others have previously reported on fabricating microfluidic channels with an aneurysm-like section [7]. However, using patient-specific computed tomography data to design these dilating geometries would increase the realism of these models.

In Chapter VI a fabrication method to locally stimulate endothelium was introduced. A proof-of-principle whole blood perfusion in an inflamed endothelialized channel was discussed. A combination of the CTA-based model, introduced in Chapter III, and a secondary channel to locally induce inflammation would be of great interest. Not only can fluid dynamical aspects of a stenotic geometry be mimicked, but additionally the inflammatory properties of the atherosclerotic site can be mimicked [8]. Besides changing the channel geometry also the microfluidic chip material could be changed. The mould

presented in Chapter VI was used for PDMS soft-lithography, but could alternatively be used to pattern biocompatible hydrogels, such as gelatin methacrylate.

7.2.3. Applications

The introduction chapter explained why models of CVD and specifically thrombosis models should be developed. Furthermore, possible applications of thrombosis-on-a-chip devices as disease models in the drug development pipeline and as a tool for personalized medicine were discussed. Not only pharmaceutical companies could benefit from these advanced models, but also academic institutions have a need for realistic thrombosis models. This need was expressed by multiple university medical centers throughout the Netherlands with whom we have started collaborations.

Daniëlle Coenen and Judith Cosemans from Maastricht University have investigated the effect of an antiplatelet therapeutic Cilostazol on washed platelets and collagen-I coated flow chambers. We have started a collaboration to investigate the effects of the addition of Cilostazol to human whole blood perfused through microfluidic channels lined with endothelial cells. A brief overview of preliminary results are shown in Appendix E. The preliminary results show a reduced platelet coverage on inflamed endothelium due to the addition of Cilostazol. It would be interesting to repeat the experiments and also investigate dose-dependent effects.

The same microfluidic model has been used to investigate sepsis with Kieu Le and Vinod Kumar of University of Groningen and University Medical Centre Groningen. Previously they have investigated the effect of various pathogens on peripheral blood mononuclear cells, that release a range of cytokines inducing inflammation and a pro-thrombotic state in endothelial cells [9]. A microfluidic model that incorporates endothelium and allows whole blood perfusion is perfect for investigating pro-thrombotic effects. Preliminary results of whole blood perfusion studies in endothelialized channels exposed to PBMC-generated cytokines are introduced in Appendix F. However, the data presented in the appendix is based on two sets of whole blood perfusion experiments. To be able to quantitatively analyze the data and extract key metrics such as platelet coverages and platelet aggregate size distributions, more experiments should be conducted.

These collaborations and the preliminary data presented in the appendices indicate that the introduced thrombosis-on-a-chip models could also be used as disease models to test antiplatelet therapeutics in a complex environment combining both activated endothelium and whole blood perfusion.

7.3. References

1. Cochrane, A.; Albers, H.J.; Passier, R.; Mummery, C.L.; van den Berg, A.; Orlova, V. V.; van der Meer, A.D. Advanced in vitro models of vascular biology: Human induced pluripotent stem cells and organ-on-chip technology. *Adv. Drug Deliv. Rev.* **2018**.
2. Gu, M.; Shao, N.Y.; Sa, S.; Li, D.; Termglinchan, V.; Ameen, M.; Karakikes, I.; Sosa, G.; Grubert, F.; Lee, J.; et al. Patient-Specific iPSC-Derived Endothelial Cells Uncover Pathways that Protect against Pulmonary Hypertension in BMPR2 Mutation Carriers. *Cell Stem Cell* **2017**, *20*, 490-504.e5.
3. Ando, J.; Yamamoto, K. Vascular Mechanobiology - Endothelial Cell Responses to Fluid Shear Stress. *Circ. J.* **2009**, *73*, 1983–1992.
4. Sinha, R.; Le Gac, S.; Verdonchot, N.; Van Den Berg, A.; Koopman, B.; Rouwkema, J. Endothelial cell alignment as a result of anisotropic strain and flow induced shear stress combinations. *Sci. Rep.* **2016**, *6*, 1–12.
5. Rustenhoven, J.; Jansson, D.; Smyth, L.C.; Dragunow, M. Brain Pericytes As Mediators of Neuroinflammation. *Trends Pharmacol. Sci.* **2017**, *38*, 291–304.
6. Halaidych, O. V.; Cochrane, A.; van den Hil, F.E.; Mummery, C.L.; Orlova, V. V. Quantitative Analysis of Intracellular Ca²⁺ Release and Contraction in hiPSC-Derived Vascular Smooth Muscle Cells. *Stem Cell Reports* **2019**, *12*, 647–656.
7. Mannino, R.G.; Myers, D.R.; Ahn, B.; Wang, Y.; Margo Rollins; Gole, H.; Lin, A.S.; Guldborg, R.E.; Giddens, D.P.; Timmins, L.H.; et al. “Do-it-yourself in vitro vasculature that recapitulates in vivo geometries for investigating endothelial-blood cell interactions”. *Sci. Rep.* **2015**, *5*, 12401.
8. Libby, P.; Ridker, P. M.; Hansson, G.K. Progress and challenges in translating the biology of atherosclerosis. *Nature* **2011**, *473*, 317–325.
9. Le, K.T.T.; Chu, X.; Jaeger, M.; Plantinga, J.A.; Matzaraki, V.; Withoff, S.; Joosten, L.A.B.; Netea, M.G.; Wijmenga, C.; Li, Y.; et al. Leukocyte-Released Mediators in Response to Both Bacterial and Fungal Infections Trigger IFN Pathways, Independent of IL-1 and TNF- α , in Endothelial Cells. *Front. Immunol.* **2019**, *10*, 2508.

Appendix

Supplementary Information

A. Supplementary Information Chapter III

Figure A-1. Thrombosis-on-a-chip fabrication process. (A) The 3D model as seen directly after printing. (B) The blue support struts were removed with a scalpel; small “pinches” were sufficient to detach the struts due to their small diameter. PDMS was then cast into the mould-box (red dotted line). (C) PDMS was cured overnight at 60 °C, resulting in a 4mm chip thickness. After curing, the PDMS was separated from the mould box by cutting the box at the edges with a scalpel and breaking it open. The removal of the 3D printed model vessel was conducted in two steps using forceps, and without the need for bending the PDMS chip during removal. (D) First, the model was gently pulled at the outlet side, where it broke at the thinnest part of the stenosis or at the heel of the outlet in case of the healthy channels. (E) Afterwards, the rest of the 3D printed construct was removed from the inlet side. (F) The resulting full 3D PDMS microfluidic chip.

Figure A-2. Collection of printing defects presented in the PIC100 constructs. Scale bars, 200 μm

Figure A-3. Microscopy images of ring-like indentations produced in the SLA printing process. (A) Stenotic section. (B) Straight section. Scale bars, 100 μm .

Figure A-4. Microscopy images of a 3D model incorporating two curves of approximately 80° , measured from the centre of curvature. (A) Close up of a print encapsulated in PDMS. (B) Close up of a cast PDMS chip after successful mould removal. Scale bar, $400\ \mu\text{m}$.

Figure A-5. Fluorescence microscopy images of a cytotoxicity measurement of HUVECs seeded in the 3D microfluidic chips. The HUVECs were stained using a live/dead staining. The number of dead cells is negligible. Scale bar, $200\ \mu\text{m}$.

Figure A-6. Finite element analysis of streamlines simulated in healthy and stenotic vessels of 67% occlusion, simulated on microfluidic scale (A) (C) and CTA scale (B) (D). Healthy (A) (B) and stenotic (C) (D) vessel geometries were compared. An input flow of 1 ml/min and a vessel diameter of 4 mm were used for the CTA scale simulations and an input flow of 0.29 ml/min and a vessel diameter of 400 μm for the microfluidic scale simulations. Both healthy geometries (A) (B) showed no sign of recirculation. The microfluidic scale simulation (C) showed minor recirculation while the CTA scale simulation showed a large recirculation area with elevated flow rates. Downstream flow directions are given in blue, stagnant flow is given in white and backflow is given in red. Scale bar (A) (C), 200 μm . Scale bar (B) (D), 2 mm.

Figure A-7. Finite element analysis of the effect of ring-like indentations with a length of 25 μm (parallel to flow direction) and height of 4 μm (perpendicular to flow direction). (A) Flow velocity profiles of a 1500 μm section, with and without ring-like indentations respectively. Scale bar, 400 μm . (B) Shear rate distribution of a channel with ring-like indentations showing a continuous scale and isolines with an equidistant spacing of 500 s⁻¹. A slightly elevated shear rate of 1500 s⁻¹ was measured on the inner part of the ridged structure. Scale bar, 25 μm . (C) Flow velocity magnitude measured from the microfluidic channel centre to the outer surface. A comparison between the smooth geometry and the ridged geometry is given, where the velocity was measured up to either the inner part or the outer part of the ridge. Inset shows a magnified view of the outer 8 μm of the channel showing almost equal flow velocities up to the outer 5 μm .

Figure A-8. Semi-quantitative platelet aggregation analysis. During perfusion the stenotic geometries revealed two aggregation mechanics, although each run depicted here revealed one predominant mechanism. (A) Healthy control revealing no platelet aggregation. (B) 55% stenotic geometry. Gradual aggregate growth around 14 minutes of perfusion and a steep drop after platelet dislodgement. Stick and slip mechanics were observed in the peak between the 15-18 minute time points (*). (C) 67% stenotic geometry. Mostly stick and slip growth, as shown by the multiple peaks and drops (*). Gradual growth of platelets was observed near the end of perfusion. (C) 67% stenotic geometry. A steep growth of platelets in the initial phase, followed by stick and slip growth (*). Missing data in (C-D, dotted lines) was intentionally left out because data was recorded out of focus or outside of region of interest. Stick and Slip regions are indicated with an asterisk (*).

B. Supplementary Information Chapter IV

Figure B-1. Computational fluid dynamics simulation with COMSOL. (A) The wall shear rate (s^{-1}) calculated for a 2 mm long section of the microfluidic channel. (B) The wall shear rate measured on a cutline on the microfluidic channel. The vertical lines indicate the border of ROI #2, and the shear rate values found in ROI #2 are equal to or greater than 95% of the maximum shear rate found in the graph.

Figure B-2. Comparison of platelet aggregate size distributions determined using the triangle threshold script or manual identification. The distribution determined with the triangle threshold is in blue while the distribution determined with the manual identification is in orange. Both aggregate size distributions are very similar, with the exception of a few bins representing larger aggregates.

Figure B-3. End-point measurements of microfluidic chips perfused with human whole blood stained with DiOC₆. (A,B) Raw data showing platelet aggregates stained with DiOC₆ in greyscale. Dashed lines show the channel walls. (C,D) Binary images produced using the triangle method. With the triangle method, platelet coverages were found to be 6.526% (C) and 4.651% (D). With the manual identification of platelets and platelet aggregates, platelet coverages were found to be 6.654% (C) and 4.597% (D). Scale bar, 100 μm .

C. Supplementary Information Chapter V

Figure C-1. Phase contrast images, 5h after seeding HUVECs. Top figure 5×10^6 cells/ml, bottom channel was seeded with 20×10^6 cells/ml. Scale bar, 150 μm .

Figure C-2. Nuclear alignment of HUVECs (orange, $n = 5$) and hiPSC-ECs (blue, $n = 5$) determined in MATLAB using the function 'regionprops' and normalized to display a cumulative length of one (MATLAB probability normalization).

Figure C-3. Confluent layers of HUVECs and hiPSC-ECs cultured in microfluidic channels in untreated and inflamed conditions. Nuclei, ICAM-1 and F-actin stained in blue, cyan and red respectively. Scale bar, 100 μm .

D. Supplementary Information Chapter VI

Figure D-1. Fabrication of a 3D-printed mold for PDMS soft lithography using the Form 2 and black resin. (A) PDMS microfluidic device (left) and 3D-printed mold (right) with 3D-printed arterial channel based on thresholded CTA-data. (B) chipholder with two inlets and outlets for tubing and pipette tip reservoirs. (C) Schematic cross-section of the “wire” and “vessel” channels separated by a collagen barrier.

Figure D-2. (A) Brightfield microscopy images of 3D-printed “vessel” channels based on thresholded CTA-data. (B-C) High magnification images of the 3D-printed vessel-like section and 2D computation fluid dynamics experiments mapping shear rate distributions. Insets show high shear regions due to 3D-printing artefacts.

E. Cilostazol as Antiplatelet Therapeutic

This appendix introduces preliminary results obtained in collaboration Daniëlle Coenen and Judith Cosemans from the University of Maastricht.

Patients affected by plaque rupture are often treated with therapeutics targeted at platelet inhibition. It is theorized that platelets in thrombi retain their activated state, thus releasing granules and their content, allowing re-occlusion to occur [1,2]. It was hypothesized that administering a therapeutic that interferes with platelet secretion of α - and δ -granules might reduce re-occlusion.

Coenen et al. have previously investigated the use of an antiplatelet therapeutic Cilostazol or Pletal®, that targets phosphodiesterase-3 effectively inhibiting: platelet adhesion, activation and secretion of α - and δ -granules [3]. Platelet granule and extracellular vesicle secretion for various concentrations of Cilostazol was measured, showing a dose-dependent effect (data not shown here). Furthermore, platelet adhesion and coverage on collagen coated flow chambers was monitored. No significant differences in platelet coverages in collagen coated flow chambers perfused with whole blood with or without Cilostazol were observed (data not shown here). It would be interesting to add a layer of complexity by replacing the collagen substrate with inflamed endothelium, which would mimic the activated endothelium in atherosclerosis [4]. Especially since activation of platelets and subsequent granule excretion results in exposure of adhesion molecules involved in interactions with activated endothelium [2].

To that end, the microfluidic models described in Chapter IV and V were lined with human umbilical vein endothelial cells (HUVECs). The HUVECs were either incubated overnight with endothelial cell growth medium (EGM-2, negative control condition) or EGM-2 with 10 ng/ml tumor necrosis factor- α (TNF- α , for control, Cilostazol and Tadalafil conditions). Re-calcified citrated human whole blood was perfused at an arterial shear rate of 1000 s⁻¹ as described in Chapter IV and V. However, DiOC₆ was used to label platelets and the period of whole blood perfusion was shortened to 12 min. At the start of each series of whole blood perfusion experiments a microfluidic channel lined with untreated endothelium was perfused with untreated whole blood as a negative control (data not shown). Microfluidic devices with inflamed endothelium were exposed to whole blood without antiplatelet therapeutics or were exposed to whole blood

with 5 μ M Cilostazol or 10 nM Tadalafil which inhibited phosphodiesterase- 3 and 5 respectively. After whole blood perfusion devices were flushed and phase contrast images were made to manually determine platelet coverage.

Figure E-1. Microfluidic channels, lined with a confluent layer of HUVECs pre-treated with 10 ng/ml TNF- α and perfused with human whole blood (A) or human whole blood with Cilostazol (B) or Tadalafil (C) for 12 minutes at an arterial shear rate of 1000 s⁻¹. (A-C) Phase contrast microscopy images of platelet adhesion in microfluidic channels. (D) Platelet surface area coverage of inflamed endothelium perfused with human whole blood (control) or human whole blood with antiplatelet therapeutics Cilostazol or Tadalafil. Scale Bars, 100 μ m.

Microfluidic devices lined with inflamed HUVECs were perfused with human whole blood for 12 minutes at an arterial shear rate. Phase contrast images of the control condition (Figure 1A, n = 6), Cilostazol condition (Figure 1B, n = 4 ,whole blood with 5 μ M Cilostazol) and Tadalafil condition (Figure 1C, n = 4 ,whole blood with 10 nM Tadalafil) show platelet adhesion in the microfluidic channels. The graph in Figure 1D shows the platelet surface area coverage for each of the conditions, where the conditions with antiplatelet therapeutics have significantly lower coverages (Students' t-test p < 0.05) compared to the inflamed control condition without therapeutics. Previous results indicated that perfusion of

whole blood with Cilostazol through flow chambers coated with collagen did not result a significant reduction in platelet surface area coverage. However, the addition of Cilostazol to whole blood prior to perfusion through microfluidic channels lined with inflamed endothelium did result in a reduction in platelet coverage. The preliminary results obtained in collaboration with the University of Maastricht nicely show that these models could be a useful addition to models used to investigate platelet biology and platelet-endothelium interactions.

E.1. References

1. Jurk, K.; Kehrel, B.E. Platelets: Physiology and Biochemistry. *Semin. Thromb. Hemost.* **2005**, *31*, 381–392.
2. Golebiewska, E.M.; Poole, A.W. Platelet secretion: From haemostasis to wound healing and beyond. *Blood Rev.* **2015**, *29*, 153–62.
3. Schror, K. The pharmacology of cilostazol. *Diabetes, Obes. Metab.* **2002**, *4*, S14–S19.
4. Libby, P.; Ridker, P. M.; Hansson, G.K. Progress and challenges in translating the biology of atherosclerosis. *Nature* **2011**, *473*, 317–325.

F. Thrombosis-on-a-Chip for Sepsis Studies

Sepsis is a disease where a pathogen or pathogen-derived factors, such as endotoxins, bacteria or fungi, find their way into the blood stream which triggers a severe immune response in the host. Peripheral blood mononuclear cells (PBMCs), *i.e.* monocytes or lymphocytes, respond to the presence of the foreign pathogens by generating cytokines.

This appendix introduces preliminary results obtained in collaboration with Kieu Le and Vinod Kumar from the University of Groningen and the University Medical Centre Groningen. They have investigated the response of PBMCs to *C. albicans*, *S. pneumonia* and Lipopolysaccharide (LPS) and have determined the response of endothelial cells to the PBMC-generated cytokines [1].

Le et al. observed upregulation of various adhesion molecules, *e.g.* ICAM-1, VCAM-1 and E-selectin [1]. These surface markers play an important role in leukocyte recruitment but also in platelet adhesion [2–6]. The microfluidic model introduced in Chapters IV and V could be used to investigate the effect of the cytokines produced by PBMCs on endothelium and ultimately how this affects platelet adhesion during whole blood perfusion.

The protocols described in Chapter IV and V were followed to fabricate microfluidic channels lined with a confluent monolayer of human umbilical vein endothelial cells (HUVECs). The pathogen specific conditions were exposed to a coinciding inflammatory cytokine mix for 4h, while refreshing the cytokine mix after 2h exposure. Channels used for positive and negative controls were exposed overnight to culture medium with or without tumor necrosis factor- α (TNF- α) respectively. Re-calcified citrated human whole blood was perfused at 1000 s^{-1} for 25 min according to the same protocols.

Figure 1 shows representative end-point measurement of platelet adhesion after 25 min of whole blood perfusion in each condition. Channels lined with endothelium exposed to PBMC-generated cytokines show elevated levels of platelet aggregation compared to the negative control. Platelet aggregate size is mapped onto the a thresholded fluorescence microscopy image with sizes ranging from $1\text{ }\mu\text{m}$ (blue) to $\geq 300\text{ }\mu\text{m}$ (red). All pathogen specific conditions show larger platelet aggregate sizes compared to the positive control. The *S. pneumonia* condition shows less single platelet adhesion in comparison to the other pathogen specific conditions and the positive control.

Figure F-1. Platelet coverage on HUVECs cultured in a microfluidic channel perfused with human whole blood, flow direction from left to right. Platelet aggregates are color coded based on aggregate surface area. Endothelial cells were allowed to reach confluency before inflammatory cytokines were introduced. After 4h exposure to the inflammatory cytokines whole blood was perfused at 1000 s^{-1} for 25 minutes. Platelets have been stained with CD41-PE and platelet coverage was observed after perfusion using fluorescence microscopy. Control experiments show that 18h exposure to 10 ng/ml TNF- α (A) results in platelet aggregation while the untreated condition (B) shows almost no platelet adhesion. The three condition with the PBMC-generated cytokines by exposure to *C. albicans* (C), *S. Pneumonia* (D) and LPS (E) all show a higher platelet coverage in comparison to the untreated control condition. Furthermore the *C. albicans* condition (C) shows long pearl-like platelet adhesion along the flow direction while the LPS condition (E) displays a scattered distribution of small platelet aggregates. An example of a microfluidic chip (F) being perfused with human whole blood by applying pressure with a syringe pump. Scale bar, 100 μm .

Previous research has shown that a large part of the inflammatory response of HUVECs is attributed to TNF- α and IL-1. Le et al. have investigated the role of each cytokine by selectively inhibiting either TNF- α or IL-1 by introducing anti-TNF- α or Anakinra respectively. Increased surface marker expression was mainly due to TNF- α (ICAM-1, VCAM-1 and E-selectin) however also IL-1 played a role (only E-selectin). When inhibiting both TNF- α and IL-1, surface marker expression decreased to baseline level. However, gene expression of

HUVECs exposed to PBMC generated cytokines without TNF- α and IL-1 showed that the inflammatory cytokine interferon (INF) also plays a role.

It would be of great interest to use PBMC-generated cytokine solutions treated with anti-TNF- α and Anakinra in the introduced microfluidic thrombosis-on-a-chip devices. These can hopefully lead to more insight into effective treatments for Sepsis patients. Furthermore, the role of the INF cytokines could be further investigated.

F.1. References

1. Le, K.T.T.; Chu, X.; Jaeger, M.; Plantinga, J.A.; Matzaraki, V.; Withoff, S.; Joosten, L.A.B.; Netea, M.G.; Wijmenga, C.; Li, Y.; et al. Leukocyte-Released Mediators in Response to Both Bacterial and Fungal Infections Trigger IFN Pathways, Independent of IL-1 and TNF- α , in Endothelial Cells. *Front. Immunol.* **2019**, *10*, 2508.
2. Bombeli, T.; Schwartz, B.R.; Harlan, J.M. Adhesion of activated platelets to endothelial cells: evidence for a GPIIb/IIIa-dependent bridging mechanism and novel roles for endothelial intercellular adhesion molecule 1 (ICAM-1), α v β 3 integrin, and GPIIb/IIIa. *J. Exp. Med.* **1998**, *187*, 329–39.
3. Coenen, D.M.; Mastenbroek, T.G.; Cosemans, J.M.E.M. Platelet interaction with activated endothelium: mechanistic insights from microfluidics. *Blood* **2017**, *130*, 2819–2828.
4. Wagner, D.D.; Burger, P.C. Platelets in Inflammation and Thrombosis. *Arterioscler. Thromb. Vasc. Biol.* **2003**, *23*, 2131–2137.
5. Wu, K.K.; Thiagarajan, P. ROLE OF ENDOTHELIUM IN THROMBOSIS AND HEMOSTASIS. **1996**.
6. van Hinsbergh, V.W.M. Endothelium—role in regulation of coagulation and inflammation. *Semin. Immunopathol.* **2012**, *34*, 93–106.

Scientific Output

Peer-Reviewed Publications

Hugo J. Albers, Robert Passier, Albert van den Berg, and Andries D. van der Meer. "Automated Analysis of Platelet Aggregation on Cultured Endothelium in a Microfluidic Chip Perfused with Human Whole Blood." *Micromachines* 10, no. 11 (2019): 781.

Amy Cochrane⁺, **Hugo J. Albers**⁺, Robert Passier, Christine L. Mummery, Albert van den Berg, Valeria V. Orlova[#], and Andries D. van der Meer[#]. "Advanced in vitro models of vascular biology: human induced pluripotent stem cells and organ-on-chip technology." *Advanced drug delivery reviews* 140 (2019): 68-77.
+Shared first-authorship, # Shared last-authorship

Pedro F. Costa⁺, **Hugo J. Albers**⁺, John E.A. Linssen⁺, Heleen H.T. Middelkamp, Linda van der Hout, Robert Passier, Albert van den Berg, Jos Malda, and Andries D. van der Meer. "Mimicking arterial thrombosis in a 3D-printed microfluidic in vitro vascular model based on computed tomography angiography data." *Lab on a Chip* 17, no. 16 (2017): 2785-2792. + Shared first-authorship

Burcu Gümüşcü⁺, **Hugo J. Albers**⁺, Albert van den Berg, Jan C.T. Eijkel, and Andries D. van der Meer. "Compartmentalized 3D Tissue Culture Arrays under Controlled Microfluidic Delivery." *Scientific reports* 7, no. 1 (2017): 3381. +Shared first-authorship

Xue D. Manz, **Hugo J. Albers**, Andries van der Meer, Harm Jan Bogaard, Robert Szulcek. "An in vitro microfluidic disease model to study whole blood-endothelial interactions and thrombus formation in real-time." *Journal of Visualized Experiments*, *In press*.

Hugo J. Albers, João P. da Silva Simão, Heleen H.T. Middelkamp, Christine L. Mummery, Robert Passier, Albert van den Berg, Valeria V. Orlova, and Andries D. van der Meer. "Human induced pluripotent stem cell-derived endothelial cells

in microfluidic channels perfused with human whole blood enable systematic studies of platelet aggregation." *In submission.*

Thomas Hoefer, Akshita Rana, Be'eri Niego, Shweta Jagdale, **Hugo J. Albers**, Elizabeth E. Gardiner, Robert K. Andrews, Andries D. van der Meer, Christoph E. Hagemeyer and Erik Westein. "Specific targeting of shear gradient activated von Willebrand Factor by the novel single-chain antibody A1 reduces occlusive thrombus formation *in vitro*." *In submission.*

Daniëlle M. Coenen, Alexandra C.A. Heinzmann, **Hugo J. Albers**, Andries D. van der Meer, Rory R. Koenen and Judith M.E.M. Cosemans. "A role for PDE3 in interaction between platelets and other cell types – working title" *In preparation*

Conference Contributions

European Society for Microcirculation – European Vascular Biology Organisation (ESM-EVBO) – 2019 (Poster presentation)

Daniëlle M. Coenen, **Hugo J. Albers**, Alexandra C.A. Heinzmann, Rory R. Koenen, Andries D. van der Meer, Judith M.E.M. Cosemans. "PDE3 and -5 Inhibition Differentially Affects Platelet Function under Hemostatic and Inflammatory Conditions"

UK Platelet Meeting - 2019 (Poster presentation)

Daniëlle M. Coenen, Alexandra C.A. Heinzmann, **Hugo J. Albers**, Andries D. van der Meer, Rory R. Koenen, Judith M.E.M. Cosemans. "A role for PDE3 in interaction between platelets and other cell types"

MicroNanoConference - 2018 (Poster presentation)

Hugo J. Albers⁺, João P. da Silva Simão⁺, Heleen H.T. Middelkamp, Christine L. Mummery, Robert Passier, Albert van den Berg, Valeria V. Orlova, and Andries D. van der Meer. "Pro-Thrombotic Response of Inflamed Human Induced Pluripotent Stem Cell-Derived Endothelial Cells." +Shared first-authorship

International Conference on Miniaturized Systems for Chemistry and Life Sciences (μ TAS) – 2018 (Poster presentation)

Hugo J. Albers⁺, João P. da Silva Simão⁺, Heleen H.T. Middelkamp, Christine L. Mummery, Robert Passier, Albert van den Berg, Valeria V. Orlova, and Andries D. van der Meer. "Human Induced Pluripotent Stem Cell-Derived Endothelial Cells in Thrombosis-on-a-Chip Devices." +Shared first-authorship

Tropo Breve Symposium in Riederalp - 2018 (Oral presentation)

Hugo J. Albers, Elder Linssen, Pedro Costa, Linda van der Hout, Heleen Middelkamp, Jos Malda, Robert Passier, Albert van den Berg & Andries van der Meer. "Using 3D-Printing to Fabricate Microfluidic Vascular Models to Mimic Arterial Thrombosis."

European Platelet Network (EUPLAN) – 2018 (Oral presentation)

Daniëlle M. Coenen, **Hugo J. Albers**, Alexandra C.A. Heinzmann, Rory R. Koenen, Andries D. van der Meer, Judith M.E.M Cosemans. "Differential effects of PDE3 and -5 on platelet function under hemostatic and inflammatory conditions"

The International Society on Thrombosis and Haemostasis meeting in Dublin - 2018 (Poster presentation)

Daniëlle M. Coenen, **Hugo J. Albers**, Andries D. van der Meer, Judith M.E.M Cosemans. "Differential effects of PDE3 and -5 inhibitors on thrombus formation on collagen versus inflamed endothelium."

Gordon Research Seminar – Hemostasis in Waterville Valley – 2018 (Poster presentation)

Daniëlle M. Coenen, **Hugo J. Albers**, Andries D. van der Meer, Judith M.E.M Cosemans. "Effects of PDE3 and -5 inhibitors on platelet activation and secretion."

Gordon Research Conference - Hemostasis in Waterville Valley – 2018 (Poster presentation)

Daniëlle M. Coenen, **Hugo J. Albers**, Andries D. van der Meer, Judith M.E.M Cosemans. "Effects of PDE3 and -5 inhibitors on platelet activation and secretion."

MicroNanoConference - 2017 (Oral presentation)

Hugo J. Albers, J. Elder Linssen, Pedro F. Costa, Linda van der Hout, Heleen H.T. Middelkamp, Jos Malda, Robert Passier, Albert van den Berg & Andries D. van der Meer. "Using 3D-Printing to Fabricate Microfluidic Vascular Models to Mimic Arterial Thrombosis."

Phaedra Fall Meeting (2nd fall meeting) – (Poster presentation & Pitch)

Hugo J. Albers, Elder Linssen, Andries D. van der Meer, Pedro F. Costa, Robert Passier, Albert van den Berg, Jos Malda. "Using Three-Dimensional Stereolithography to Fabricate Blood Vessel Models for Thrombosis-on-a-Chip Applications."

International Conference on Miniaturized Systems for Chemistry and Life Sciences (μ TAS) – 2017 (Poster presentation)

Hugo J. Albers, J. Elder A. Linssen, Pedro F. Costa, Heleen H.T. Middelkamp, Linda van der Hout, Jos Malda, Robert Passier, Albert van den Berg, Andries D. van der Meer. "Using 3D-Printing to Fabricate a Microfluidic Vascular Model to Mimic Arterial Thrombosis."

International Conference on Miniaturized Systems for Chemistry and Life Sciences (μ TAS) – 2017 (Poster presentation)

Burcu Gümüşcü Sefünc, **Hugo J. Albers**, Albert van den Berg, Jan C.T. Eijkel, Andries D. van der Meer. "A Gut-on-a-Chip Study: Enabling On-Demand Manipulation of the Outer Cell Microenvironment in a Multicompartmental 3D Culture Array."

TERMIS Europe - 2017 (Poster presentation)

Pedro F. Costa, J. Elder A. Linssen, **Hugo J. Albers**, Robert Passier, Albert van den Berg, Jos Malda, Andries D. van der Meer. "Biofabrication of a Stenotic Vessel Model."

International Organ-on-Chip Symposium in Lausanne - 2017 (Poster presentation)

Hugo J. Albers, J. Elder A. Linssen, Pedro F. Costa, Jos Malda, Robert Passier, Albert van den Berg and Andries D. van der Meer. "Using Three-Dimensional

Stereolithography to Fabricate a Microfluidic Vascular Model to Mimic Arterial Thrombosis."

Biofabrication conference - 2016 (Poster presentation)

Pedro F. Costa, J. Elder A. Linssen, **Hugo J. Albers**, Robert Passier, Albert van den Berg, Jos Malda, Andries D. van der Meer. "3D Biomanufacturing of a Stenotic Vessel Model."

MicroNanoConference - 2016 (Poster presentation)

Hugo J. Albers, J. Elder A. Linssen, Andries D. van der Meer, Pedro F. Costa, Robert Passier, Albert van den Berg, Jos Malda. "Using Three-Dimensional Stereolithography to Fabricate Blood Vessel Models for Thrombosis-on-a-Chip Applications."

Nanocity conference – 2016 (Poster presentation)

Burcu Gumuscu, **Hugo J. Albers**, Andries D. van der Meer, Albert van den Berg, Jan C.T. Eijkel. "Can We Fabricate Multicompartmental Gut-on-Chip Platforms Using 3D Hydrogel Micropockets?"

Samenvatting

Hart- en vaatziekten zijn wereldwijd de voornaamste reden voor sterfte (verantwoordelijk voor 1/3 van alle sterftegevallen), waar het merendeel wordt veroorzaakt door een hart- of een herseninfarct. Aan de grondslag van deze aandoeningen ligt trombose, oftewel het ontstaan van bloedpropjes in bloedvaten. Er is al veel onderzoek gedaan naar deze aandoeningen en naar therapeutische strategieën om deze aandoeningen te voorkomen of genezen. Echter zijn huidige pre-klinische onderzoeksmethoden vaak niet in staat om een correcte voorspelling te doen over de werkzaamheid en toxiciteit van medicatie in een klinische setting, oftewel toegepast in patiënten. In **Hoofdstuk I** werd een overzicht gegeven van de recente ontwikkelingen op het gebied van orgaan-op-
een-chip modellen specifiek gericht op onderzoek naar trombose. Naar aanleiding van dit overzicht werd er geconcludeerd dat er een grote behoefte is aan microfluidische trombosemodellen met realistische geometrieën, persoon-specifieke cellen en methodes om een lokale ontsteking na te bootsen. Tevens zou een automatische data analyse methode helpen bij het betrouwbaar verwerken van grote hoeveelheden data. Een overzicht van recentelijke ontwikkelingen in het gebruik van stamcel afgeleid materiaal voor onderzoek naar hart- en vaatziekten werd gegeven in **Hoofdstuk II**. Daarnaast werden er verschillende microfluidische modellen van bloedvaten besproken. Tevens werden de voordelen die te halen zijn wanneer stamcel afgeleid materiaal in complexe microfluidische kanalen wordt gekweekt besproken. In **Hoofdstuk III** werd een 3D-geprinte microfluidische chip, gebaseerd op computertomografie-angiografie data van een kransslagader, geïntroduceerd. Mallen met en zonder stenotische geometrieën werden geprint en gebruikt om microfluidische chips te fabriceren. Deze 3D kanalen werden bekleed met menselijke endotheelcellen en gebruikt voor volbloed perfusie experimenten. Door hoge schuifspanningen in de stenotische geometrie ontstonden stolsels bij de stenose, terwijl in de gezonde geometrieën geen trombose te zien was. In **Hoofdstuk IV** werd een beeldanalyse methode geïntroduceerd om aggregatie van bloedplaatjes op endotheelcellen in microfluidische kanalen te analyseren. Deze methode bleek geschikt te zijn om breed uiteenlopende data, *i.e.* zowel kanalen met onbehandelde als ontstoken endotheelcellen te verwerken. Tevens bleek deze methode een hoge sensitiviteit, specificiteit en nauwkeurigheid te hebben. Deze analyse methode is vervolgens

gebruikt in **Hoofdstuk V** voor een kwantitatieve analyse van aggregatie van bloedplaatjes op primaire endotheelcellen en stamcel afgeleide endotheelcellen. Deze werden beide gekweekt in microfluidische kanalen. Er werd geen significant verschil in de mate van aggregatie gevonden voor beide vaatwandcellen. Tevens was een plaatjesremmer 'Eptifibatide' aan het bloed toegevoegd en waren beide door microfluidische kanalen met stamcel afgeleide vaatwandcellen gepompt. Dit resulteerde in significant lagere bloedplaatjes adhesie. In **Hoofdstuk VI** werd een 3D-geprinte microfluidische chip geïntroduceerd om in een met endotheel bekleed kanaal lokaal een ontsteking te induceren. De toxiciteit van het 3D-geprinte materiaal was getest alvorens een volbloed perfusie experiment bewees of het mogelijk was een ontsteking te induceren. Na een samenvatting en outlook in **Hoofdstuk VII** zijn er verscheidene supplementaire figuren en twee korte beschrijvingen van samenwerkingen met twee universitair medisch centra in de **Appendix** gegeven.

Contributions

The work described in this thesis is the result of a combined effort of multiple people, of course my direct supervisors: Andries van der Meer, Albert van den Berg and Robert Passier have played an important role in all chapters. The review presented in chapter II was written in collaboration with Amy Cochrane, Valeria Orlova and Christine Mummery from the LUMC. The experimental work in Chapter III was done in collaboration with Elder Linssen, Pedro Costa and Jos Malda from the UMC. Heleen Middelkamp helped setup the first blood runs and results of Linda van der Hout, acquired during her MSc assignment, were incorporated in the chapter. The automated image analysis script presented in Chapter IV is based on the work of Zach et al. which was incorporated in a MATLAB script by Bernard Panneton. The work presented in Chapter V was the result of a collaboration between Valeria Orlova and Christine Mummery of the LUMC who provided us with hiPSC-ECs. Heleen Middelkamp provided us with protocols on how to incorporate hiPSC-ECs in microfluidic channels which was optimized during the MSc assignment of João Simão who also delivered a proof-of-principle of the study. Johan Bomer provided us with the wafers used for microfluidic chip fabrication. The work presented in Chapter VI is the combined result of the internships and final assignments of Simone ten Den, Kelly Klaassen and Meike Große-Elshoff. Surface functionalization protocols were provided by Wesley Buijsman and Joshua T. Loessberg-Zahl, who also helped with the 3D-printing of the moulds.