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LETTER

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Influence of multiple stenoses on thrombosis formation: an in vitro study

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Abstract

Multiple lesions in the same vessel is one of the most common situations found in patients suffering from cardiovascular diseases, this complicates not only the assessment of the severity of each one but also their treatment. To date, the effect of multiple stenoses on different parameters has been simulated by numerical studies. Few others have implemented in vitro platforms for their investigation. However, visualization of thrombosis formation in this kind of lesion is still needed. This in vitro study monitors the formation of thrombus inside microchannels having one, two, and three stenoses. Whole blood was perfused through each channel at high shear rates (> 12,000 s^{-1}), generating thrombosis. Flow changes across each lesion as well as the final percentage of aggregations were monitored. Thus, the location where total occlusion could be produced was found to be the first stenosis for all the cases. Less flow reaching the second and third stenoses was observed which demonstrates that aggregations were growing at the first one. This was verified by measuring the percentage of aggregations at the end of the test.

Keywords: Thrombosis, Multiple stenoses, Platelets, Whole blood, Microfluidics, In vitro model, Lab-on-a-chip

Introduction

Cardiovascular diseases (CVDs) are the leading cause of death worldwide, it has been reported that it takes even more lives every year than all types of cancer and chronic lower respiratory disease together [1]. Some of the consequences of CVDs resulting in death are coronary heart disease, strokes, and heart attacks. These are mainly caused by blood clots in the arteries blocking the normal supply of blood which is known as thrombosis. Thrombosis develops when an atherosclerotic plaque within the artery suffers an injury allowing the interaction between fats and cholesterol inside the plaque with blood. Thus, leading to the growth of a thrombus composed of red blood cells, platelets, and fibrin. This process is known as thrombogenesis [2].

Plaques in arteries can be different in their geometry, size, and length. They can also, be present as single and multiple (or serial) lesions. Compared to single stenosis, fluid dynamics in serial stenoses is complex, therefore an accurate diagnosis and treatment are still a medical challenge [3]. Keeley et al. [4] found that 69% of patients with ST-segment elevation myocardial infarction had multiple complex stenoses, of which 26% had two stenoses and 17% had three stenoses. According to the results of Goldstein et al. [5] patients with serial stenoses have a higher risk of recurrent ischemia as well as higher mortality compared to patients with single stenosis.

High wall shear rates play an important role in coronary thrombosis development [6]. They are responsible for starting the mechanism for platelets' aggregation [7]. An in vivo study supported that platelet aggregations develop not only by soluble agonists but also by shear stress gradients [8]. Wall shear rate is the result of the wall shear stress divided by the viscosity of the fluid [9]. Numerical studies have simulated the flow dynamics present in serial stenoses, demonstrating that wall shear

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stress increases as the percentage of stenosis increases [10]. However, the presence of multiple stenoses has more impact on flow impairment than single stenoses with the same degree [11, 12].

While assessing multiple stenoses, one of the most important parameters is pressure drop. The pressure gradient is used mainly when diagnostic techniques such as fractional flow reserve (FFR) are measured. FFR index helps to evaluate the lesion severity and to decide on treatment [13]. Pressure drop has been found to vary and depend on the number of stenoses in a vessel [10, 14]. Also, a larger pressure gradient is produced when the distance between proximal and distal stenoses increases [15]. It has been found that post-stenotic sections are prone to develop particle aggregations due to recirculation zones and flow separation [14], which could lead to thrombosis development.

Through these studies, prothrombotic sites can be estimated, however, in vitro experiments proving these predictions in thrombosis formation are still lacking. This study demonstrates the effect that the number of stenoses has on thrombogenesis, the location of thrombus formation, and how multiple stenoses increase the risk of thrombosis and total occlusion. These results contribute to a broader understanding of how thrombosis might develop in multiple stenoses. Moreover, thrombus formation monitoring and final aggregations measurement allow the determination of the lesion prone to occlusion.

Fabrication

A microfluidic chip with channels having one (a), two (b), and three stenoses (c) was designed. A clinical severe narrowing of 85% was chosen for all of the lesions. As the purpose was to find the effect of the number of stenoses, all of them were designed the same. The geometry of the stenosed channels was concentric. The diameter of the channel was 600 µm while the diameters in the stenotic parts were 90 µm. The distance between lesions was set as 5 mm (Fig. 1).

A silicon wafer was patterned by photolithography and master molds were created by soft lithography. Chips were made of PDMS 10:1, using the thermal air expansion method developed in our laboratory [16]. This method allows the fabrication of self-aligned elliptical channels, as the intention is to mimic the shape of human arteries.

Collagen type I from rat tail (1 mg/ml, Sigma-Aldrich, Korea) was used for creating a prothrombotic coating inside the channel. Channels were incubated overnight at room temperature and rinsed with PBS 1X before experiments [17].

Experiment

Whole blood was drawn from one volunteer and collected in a bag containing CPDA-1 anticoagulant; before use, it was recalcified with the proper amount of $CaCl_2$ 0.25 M. Blood was perfused through the channels using a flow rate of 3 ml/h controlled by a syringe pump, connected by tubing from the syringe to the inlet of the channel. Blood flow and thrombus formation were observed by an optical microscope (DMSZ7, Sunny Optical, China). Pressure drop was monitored by two pressure sensors (ABP series, Honeywell, US) placed at the inlet and outlet. The setup and chip used for the experiment are shown in Fig. 2.

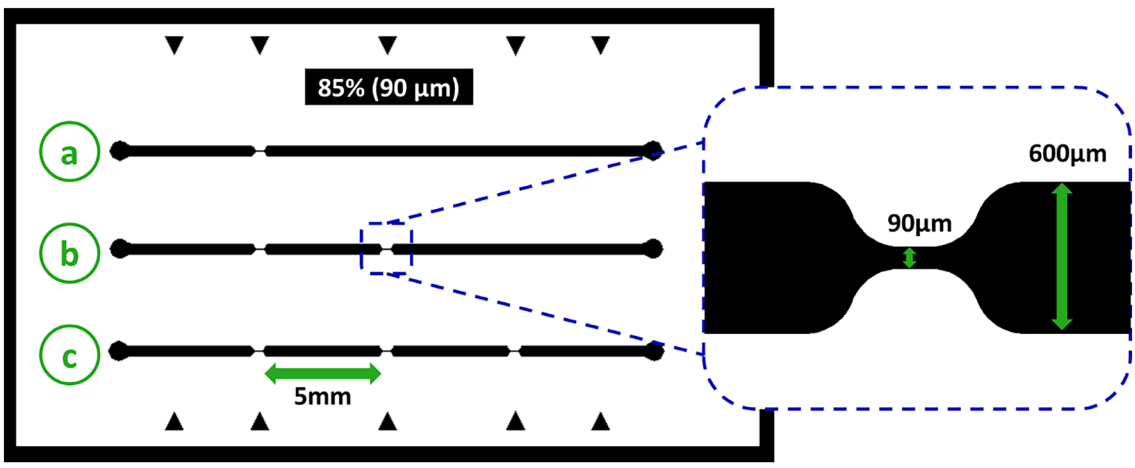

Fig. 1 Microfluidic chip designed for the experiment. Channels with one (**a**), two (**b**), and three (**c**) lesions. Zoomed part shows the stenosis geometry and sizes

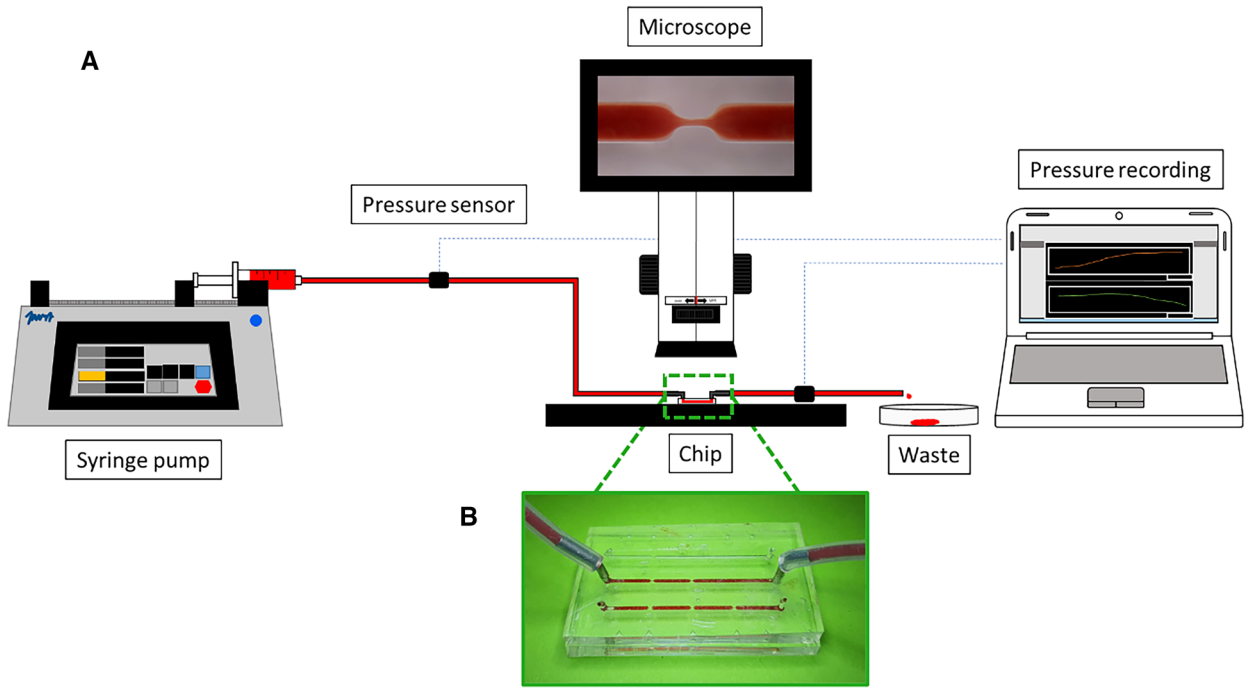

Fig. 2 **A** Setup for monitoring thrombosis formation. **B** PDMS microfluidic chip

Analysis

To determine how much a second and/or third stenosis is affected by thrombosis, each lesion was observed separately. Video recordings were used for analysis. Frames at each time of interest were obtained, and images were processed using ImageJ software. A mask was used for defining the area to be analyzed and the region of interest was cropped. Then, clear areas were determined by color thresholding using the HSB (hue-saturation-brightness) method. The size of the whole region of interest and clear areas were obtained, and percentages were calculated.

For determining the flow reaching each stenosis, images during perfusion were used and clear area percentages were calculated for the first 600 s. Normally, when clear areas appear, the area indicates the existence of a thrombus [18]. However, during our control experiments, three different phenomena were observed that might be the reason explaining these clear areas. As is shown in Fig. 3, one of the causes of the color difference is the formation of aggregations, they appear like clots with light color, as they are mainly composed of platelets. Also, when aggregations appear, the flow is deviated from its normal path, creating a light-colored string. The third reason is the phenomenon known as "cell-free layer" (CFL). When the blood flows through the microvessels, red blood cells tend to gather in the middle of the flow leaving a layer of plasma without

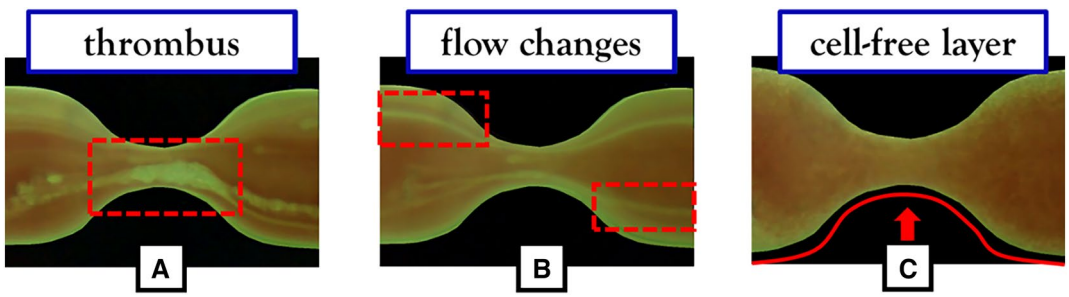

Fig. 3 Three different reasons for clear areas in blood flow. **a** Formation of thrombus, **b** flow changes due to thrombus formation, **c** cell-free layer

cells on the edges. This cell-free layer shows a different color from the usual blood color.

As the appearance of clear areas could be ambiguous, another measurement was taken for verifying the risk of occlusion of each stenosis. Images were taken at the end of the experiment when blood was not flowing anymore, and they were also considered in the analysis. The percentage of clear areas at each lesion was calculated and they were declared to be aggregations. As flow was already stopped two of the possible reasons for clear areas were discarded: flow changes and cell-free layer.

Results and discussion

Clear areas percentage was measured in the channel with only one stenosis. Through this analysis, changes at the stenotic part during blood flow were observed, such as aggregations, and lysis or embolism. Figure 4 shows the percentage of clear areas at some points during 600 s of perfusion where these changes were evident.

When blood started flowing, an increment in the clear area was observed, which was due to aggregations forming inside the channel. The clear area was then reduced meaning that the clot was dissolved and therefore, blood occupied more space in the channel. Reaggregation was also observed at 600 s.

Same observation and analysis were made for second and third stenoses. Contrary to what happened in the first one, at these lesions, aggregations were not observed. Instead, a constant increment in clear area percentages was noticed (Fig. 5). Meaning that blood flow reaching these stenoses was decreasing. Aggregations being formed in the first stenosis could explain these changes. Thrombus growth at the first lesion causes higher resistance to flow; therefore, less flow reaches the second and third stenosis.

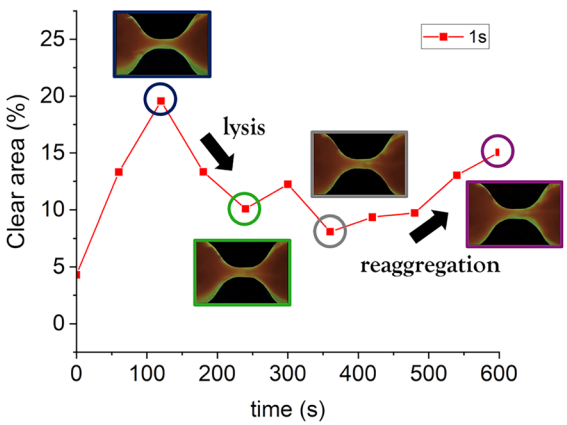

Fig. 4 Flow changes during blood perfusion in the channel with one stenosis

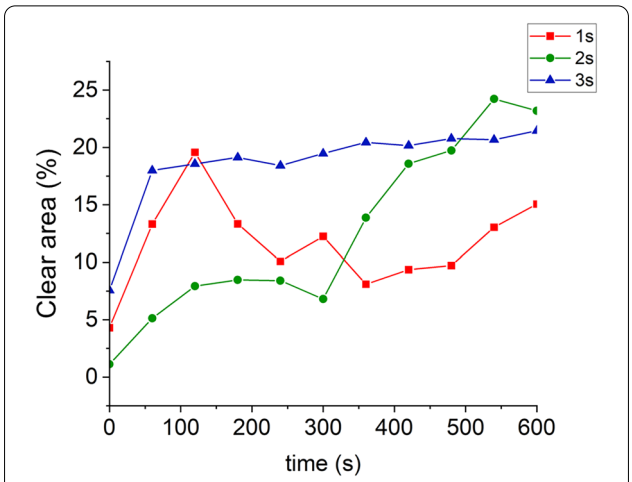

Fig. 5 Clear area percentages trend at second (green) and third (blue) stenoses compared to first stenosis (red) behavior

For confirming the results found during perfusion, the aggregation percentage was measured at the end of the experiment, these results are shown in Fig. 6. Aggregation percentages were analyzed at each stenosis in the three channels and the highest percentage was found at the first stenosis in all the cases. Thus, demonstrating that the first stenosis was the cause of the changes occurring in the second and the third one. Even if the second and the third stenosis also showed a considerable percentage of aggregations, the first one showed the most.

Results obtained through this study, give us an insight into the development of thrombosis in vessels with multiple stenoses. Thrombosis formation at high shear rates was confirmed. Aggregation and embolism or lysis were observed at the first stenosis during blood perfusion, which demonstrates thrombus instability, however, total occlusion was not reached.

It was observed that in a case where all the stenoses have the same severity, the responsible for total occlusion would be the first one. This could be explained because even if shear rates are almost the same at each lesion, the first lesion is the first one having contact with blood, therefore, the thrombosis mechanism starts there. Thrombus in second and third stenoses was not observed, however, less flow was found to reach them. Aggregations in a lower percentage were also found in these lesions.

The behavior of severe stenosis of 85% was confirmed through this study, however, some limitations were also encountered. First, our design shows an ideal case where all the stenoses have the same degree of severity, and all of them have been reached by the collagen product of a rupture somewhere. Besides collagen coating not being localized, soluble collagen was used

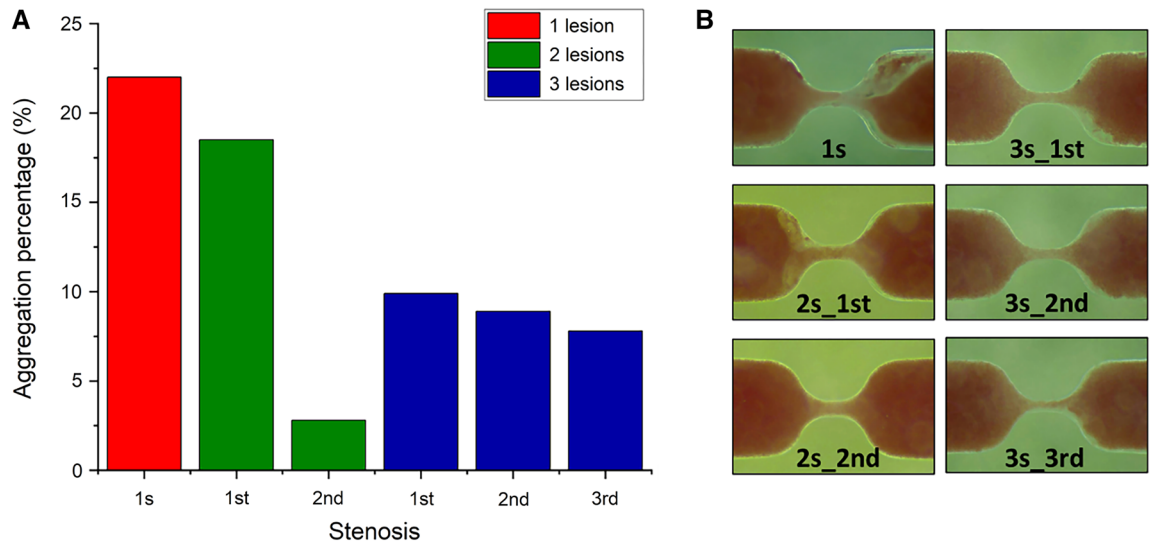

Fig. 6 **A** Percentage of aggregations at each lesion in channels with one, two, and three stenoses. **B** Picture of each stenosis, where aggregations can be observed in a lighter color

for these experiments, which contains less von Willebrand factor protein, which is necessary for arterial thrombosis development [19]. This might be the reason why total occlusion was not reached and aggregation percentages were not too significant. Also, a limited number of samples was used, therefore, findings need to be verified further. In terms of analysis, clear areas were defined by the author based on the observations. However, normally the flow should be recovered after the divergent. One explanation for this could be the separation of blood components, as it happens with the cell-free layer (CFL). Clear areas might be plasma, as it has been found that geometry variations of microfluidics enhance CFL. This has been used for blood plasma separation [20]. Regarding the monitoring, an optical microscope was utilized, so the resultant image is 2D, which does not allow for observing the volume of the aggregations, thus, the area was measured. Monitoring was made separately for all the lesions; this could be improved later by implementing simultaneous monitoring for having a wider observation of thrombus and blood flow behavior through all the stenoses.

This study demonstrated one of several conditions commonly found in patients with this disease. A similar fabrication method, experiment setup, and perhaps evaluation technique could be applied to more complex coronary artery lesions in the future. For example, atherosclerotic plaques within bifurcation lesions, which is one of the most difficult lesions to diagnose and treat. Furthermore, the device could be also lined with endothelial cells to provide the biochemical effect and make it more similar to a living artery.

Conclusion

Thrombosis development in multiple stenoses was investigated using a microfluidic in vitro model. The formation of thrombus at severe stenosis (85% narrowing) and high shear rates was confirmed. Flow reduction through the following stenoses was observed. Total occlusion was not reached due to unexpected lysis. At the beginning, it was expected that embolus coming from the first stenosis could generate occlusion in the following stenoses, but this was not observed. Clots grew in all the stenoses; however, the highest aggregation percentage was found in the first stenosis. Based on our results, the first stenosis is the most prone to total occlusion, thus, it should be treated earlier.

Abbreviations

CVDs: Cardiovascular diseases; FFR: Fractional flow reserve; HSB: Hue-saturation-brightness; CFL: Cell-free layer.

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Not applicable.

Author contributions

WTP supervised the findings of this work and reviewed the manuscript. CJS, HBFM, HDH, JHK, and PHJ fabricated the device. JHC, WTP, CJS, HBFM, and HDH designed the scope and performed the experiments. HBFM drafted the manuscript. WTP read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Declarations

Competing interests
The authors declare that they have no competing interests.

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References

1. American Heart Association (2021) Heart disease and stroke statistics update fact sheet at-a-glance; 2021. Circulation 626-44
2. Chang JC (2018) Thrombogenesis and thrombotic disorders based on "two-path unifying theory of hemostasis": philosophical, physiological, and phenotypical interpretation. Blood Coagul Fibrinol 29:585–595. https://doi.org/10.1097/MBC.0000000000000769
3. Kumbhani DJ, Bhatt DL (2016) Fractional flow reserve in serial coronary artery stenoses. JAMA Cardiol 1:359–360. https://doi.org/10.1001/jamacardio.2016.0219
4. Keeley EC, Mehran R, Brener SJ, Witzenbichler B, Guagliumi G, Dudek D, Kornowski R, Dressler O, Fahy M, Xu K, Grines CL, Stone GW (2014) Impact of multiple complex plaques on short- and long-term clinical outcomes in patients presenting with ST-segment elevation myocardial infarction (from the Harmonizing Outcomes with Revascularization and Stents in Acute Myocardial Infarction [HORIZONS-A]. Am J Card 113:1621–1627. https://doi.org/10.1016/j.amjcard.2014.02.016
5. Goldstein J, Demetriou D, Grines C, Pica M, Mazen S, O'Neill W (2000) Multiple complex coronary plaques in patients with acute myocardial infarction. N Engl J Med 343:915–922. https://doi.org/10.1056/NEJM200009283431303
6. Liu ZL, Bresette C, Aidun CK, Ku DN (2022) SIPA in 10 milliseconds: VWF tentacles agglomerate and capture platelets under high shear. Blood Adv 6:2453–2465. https://doi.org/10.1182/bloodadvances.2021005692
7. Casa LDC, Ku DN, Woodruff GW (2017) Thrombus formation at high shear rates. Annu Rev Biomed Eng 19:415–433. https://doi.org/10.1146/annurev-bioeng-071516
8. Nesbitt WS, Westein E, Tovar-Lopez FJ, Tolouei E, Mitchell A, Fu J, Carberry J, Fouras A, Jackson SP (2009) A shear gradient-dependent platelet aggregation mechanism drives thrombus formation. Nat Med 15:665–673. https://doi.org/10.1038/nm.1955
9. Casa LDC, Deaton DH, Ku DN (2015) Role of high shear rate in thrombosis. J Vasc Surg 61:1068–1080. https://doi.org/10.1016/j.jvs.2014.12.050
10. Kabir MA, Alam MF, Uddin MA (2021) Numerical simulation of pulsatile blood flow: a study with normal artery, and arteries with single and multiple stenosis. J Eng Appl Sci 68:1–15. https://doi.org/10.1186/s44147-021-00025-9
11. Huh HK, Choi WR, Ha H, Lee SJ (2016) Flow characteristics around proximal and distal stenoses in a series of tandem stenosed vessels. J Biomech 49:2960–2967. https://doi.org/10.1016/j.jbiomech.2016.07.014
12. Diken AI, Yalçinkaya A, Hanedan O, Günertem E, Demir E, Erol E (2017) Effect of tandem lesions on haemodynamic parameters: an experimental study. Kardiol Pol 75:1315–1323. https://doi.org/10.5603/KP.a2017.0163
13. Achenbach S, Rudolph T, Rieber J, Eggebrecht H, Richardt G, Schmitz T, Werner N, Boenner F, Möllmann H (2017) Performing and interpreting fractional flow reserve measurements in clinical practice: an expert consensus document. Interv Cardiol Rev 12:97–109. https://doi.org/10.15420/icr.2017:13:2
14. Bernad SI, Bernad ES, Craina M, Sargan I, Totoran A, Brisan C (2012) Particle depositions and related hemodynamic parameters in the multiple stenosed right coronary artery. J Clin Med Res 4:177. https://doi.org/10.4021/jocmr843w
15. Liu B, Zheng J, Bach R, Tang D (2017) Influences of flow parameters on pressure drop in a patient specific right coronary artery with two stenoses. In: International conference on computer science and applications. Springer, London, pp 56–70
16. Nguyen TQ, Park WT (2015) Rapid, low cost fabrication of circular cross-section microchannels by thermal air molding. In: Proceedings of the IEEE international conference on micro electro mechanical systems (MEMS). Institute of Electrical and Electronics Engineers Inc., New York, pp 348–350
17. Li M, Hotaling NA, Ku DN, Forest CR (2014) Microfluidic thrombosis under multiple shear rates and antiplatelet therapy doses. PLoS ONE 9:e82493. https://doi.org/10.1371/journal.pone.0082493
18. van Rooij BJM, Závodszky G, Hoekstra AG, Ku DN (2021) Haemodynamic flow conditions at the initiation of high-shear platelet aggregation: a combined in vitro and cellular in silico study. Interface Focus 11:20190126. https://doi.org/10.1098/rsfs.2019.0126
19. Bernardo A, Bergeron AL, Sun CW, Guchhait P, Cruz MA, Lo JA, Fong JD, Pez LJJ-F Von Willebrand D (2004) Von Willebrand factor present in fibrillar collagen enhances platelet adhesion to collagen and collagen-induced platelet aggregation. J Thromb Haemost 2:660–669. https://doi.org/10.1111/j.1538-7836.2004.00661.x
20. Faivre M, Abkarian M, Bickraj K, Stone HA (2006) Geometrical focusing of cells in a microfluidic device: an approach to separate blood plasma. Biorheology 43:147–159

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