DEVELOPMENT OF AN ASYMMETRIC STENOSIS MODEL TO STUDY THE INTERACTIONS BETWEEN PLATELETS AND ENDOTHELIAL CELLS

Leonie Rouleau *, Monica Farcas *, Jean-Francois Theoret ~, Rosaire Mongrain PhD **,***, Jean-Claude Tardif MD ~, Eric Thorin PhD ~, Yahye Merhi PhD ~, Richard L. Leask PhD *,***

*Department of Chemical Engineering, McGill University, Montreal, Canada
**Department of Mechanical Engineering, McGill University, Montreal, Canada
*** Montreal Heart Institute, Montreal, Canada

leonie.rouleau@mail.mcgill.ca

Abstract : Coronary artery disease (CAD) is the leading cause of death and hospitalization in the World. Hemodynamic forces are known to play a key role in the development of CAD and these forces have been linked to endothelial cell dysfunction and platelet activation. To better understand the response of platelets and endothelial cells to complex hemodynamic forces, we have developed a three dimensional cell culture model in the shape of an idealized asymmetric vascular stenosis. Endothelial cells have been grown up to confluency within the stenosis models. When subjected to laminar flow at physiological shear stress, shear rate and Reynolds numbers, they have been shown to align with flow direction and to keep their polygonal shape in the low shear stress recirculation zone. Platelet adhesion on fibronectin substrate in the same transparent, compliant and non-toxic models has been observed. This innovative technique provides a more realistic model to study endothelial cell response to shear stress and to characterize interactions between endothelial cells and platelets in a truly three-dimensional dynamic environment.

Introduction

Coronary artery disease (CAD) is the leading cause of death and hospitalization in North America [1]. Atherosclerotic plaques, which lead to stenosis, develop at focal regions where blood flow is disturbed, such as regions of bifurcations and curvatures [2]. In addition, it is clear that once an obstruction has developed, blood flow is further disturbed and hemodynamic factors play an important role as the stenosis continues to develop [3]. Blood flow in stenotic vessels creates a complex pattern of hemodynamics in that shear stress increases dramatically in the throat of the stenosis and then decreases very rapidly in the post stenotic recirculation region [4].

Thrombosis is frequently associated with arterial coronary stenosis and can potentially lead to unstable angina, myocardial infarction, and sudden ischemic death [5]. Thrombosis results from a disruption in the balance between the complex network of procoagulant and anticoagulant factors through the interaction between blood components, vessel wall and hemodynamic forces [6].

Endothelial cells are known to play a leading role in the development of CAD. They regulate vascular tone, inflammation, thrombosis, and vascular remodeling. They are subjected to forces which include hydrostatic pressure, cyclic strains and wall shear stresses [7]. Their structure and function [8-14] are determined, at least in part, by wall shear stress. It is now believed that wall shear stress alone can alter endothelial cell phenotype to one that promotes lesion and thrombus formation.

Platelets are anucleated blood cells derived from the cytoplasm of megakaryocytes that reside in bone marrow [15,16]. They are involved in physiologic homeostasis and pathologic thrombosis [16]. Adhesion of platelets to exposed subendothelial tissue following vascular injury is known to cause stenosis formation [5]. Dynamic models have studied the activation, adhesion and aggregation of platelets in different shear stress conditions [17,4]. However, few studies provide a complete description of the shear stresses in the models, consider interactions with other cells and most studies to date subject platelets to non-physiological hemodynamic forces [18] and use non-realistic geometries.

Very few *in vitro* endothelial cell flow experiments have been reported in compliant three dimensional stenosis geometries and none have considered the interaction with other cell types such as platelets. The objective of this study was to design a transparent and compliant idealized stenosis model, to characterize the response of the endothelial cells and platelets to shear stress and to study the interactions between endothelial cells and platelets in a well defined complex shear field.

Materials and Methods

Models: For a Newtonian fluid, shear stress is proportional to the viscosity of the fluid and to the

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speed at which adjacent layers of fluid slide past each other, i.e. the strain rate as seen in equation 1.

$$\tau = \mu \frac{dv}{dr} = \mu\gamma \quad (1)$$

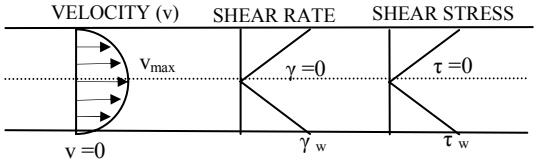

Figure 1: Laminar fully developed blood flow in a tubular vessel

The wall shear stress (τ) and the wall shear rate (γ) (equations 2 and 3) in a laminar steady flow tubular vessel, assuming an incompressible, rigid and Newtonian fluid, can be calculated as:

$$\tau = \frac{32\mu Q}{\pi D^3} \quad (2)$$

$$\gamma = \frac{32Q}{\pi D^3} \quad (3)$$

where μ is the dynamic viscosity, Q the volumetric flow rate and D the diameter [6]. This formula can give a reasonable estimate of the mean wall shear stress in straight arteries. The characterization of shear stress in complex geometries such as those seen *in vivo*, requires experimental work or numerical modeling which becomes even more complex when the pulsatile nature of blood flow is taken into account.

The geometric model used in this study is an idealized model of a coronary artery with a 50 % eccentric stenosis (flow reduction as compared to sane reference segment) with a bi-Gaussian profile. This model is a simplification of the possible complexity of lesion configurations. The velocity vectors in this geometry have been previously reported by our group using particle image velocimetry (PIV). The velocity data was used to determine the spatial distribution of shear stresses [19].

The culture models were built using a non-toxic, transparent and compliant material, Sylgard 184® (Dow Corning, PA, USA). It an innner diameter of 1/8 inch and a length of 3 inch. The fluid parameters were non-dimensionalized in order to reproduce physiological perfusion. For steady flow, these parameters include consideration of the Reynolds number (Re), shown in equation 4, that represents the ratio of the inertial over viscous forces:

$$\mathrm{Re} = \frac{\rho \bar{V} D}{\mu} \quad (4)$$

where ρ is the density and $\bar{V}$ the average inlet velocity [6]. The Re used was based on the hypothesis that the heart pumps approximately 5.5 L/min, that 5 % of this flow goes in three coronary arteries [20] and that the typical artery diameter is 3 mm [21]. The blood density taken in the calculations is 1050 kg/m^3 and the blood viscosity 0.0035 kg/(m.s) or 3.5cP. The corresponding physiological Re is 194. This result was used to calculate the flowrate necessary to obtain the same shear stress and shear rate profile at the entry of the models. The entrance length (l_e) required to obtain a parabolic velocity profile at the entry of the model as the flow is laminar is given by equation 5

$$l_e = 0.06 * \mathrm{Re} * D \quad (5)$$

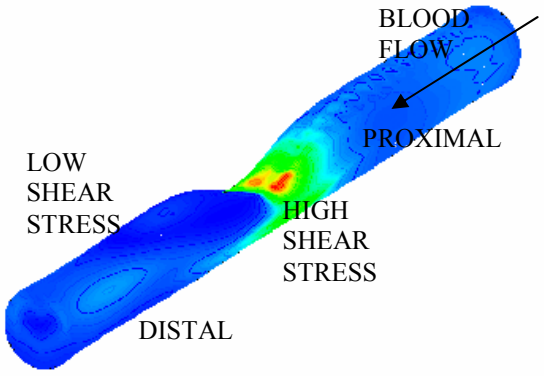

Figure 2: Shear stress patterns in the asymmetric stenosis model

Endothelial Cells: Human abdominal aortic endothelial cells (HAAE, CRL-1730) were obtained from American Type Culture Collection (ATCC, USA). Subsequent cultivation was maintained at 37° C in a humidified atmosphere (5% CO_2 in air) by using endothelial cell growth medium (C-22010, Promocell, Germany) supplemented to 10% FBS (Invitrogen Corporation, Canada). At confluency, cultures were rinsed with PBS 1X (BP399-1, Fisher Scientific, USA) and harvested with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution (MT250251CI, Fisher Scientific, Canada) and the cells were split at a ratio of 1:4 for inoculation. Each harvest and split is referred to as one passage. Fourth and fifth passages were used for perfusion experiments and static controls.

The models were hydrophilized using 75% sulphuric acid (A300-500, Fisher Scierntific, USA) for 7 minutes. They were then boiled using deionized water for 30 minutes and coated using fibronectin at a concentration of 40 μg/mL (F0895, Sigma-Aldrich, Canada) for 24 hrs at 37° C. In order to ensure even coating of all sides of the model, a tube rotator, rotating at 8 rpm (Barnstead/Thermolyne, Fisher Scientific, Canada) was used. Solutions were aspirated off and the models washed with complete media once before the seeding started.

Endothelial cells were seeded four times, at a seeding density of 3 X 10^5 cells/mLon all sides at 5 minutes interval (Beckman Coulter Counter). The tubes were rotated by 90° between seedings. Each seeding attached cells to a different side of the tube. Experiments were initiated after a cultivation period of 24 hrs, i.e. when a confluent monolayer had been

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established. The connectors at both ends of the models were used to install the models in the flow loop.

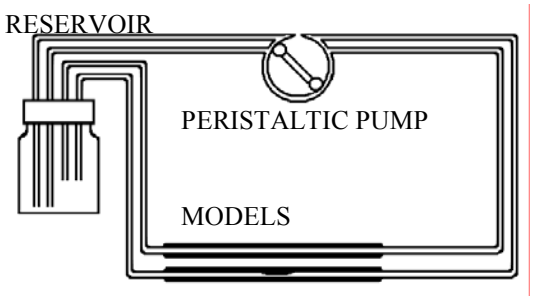

Figure 3: Schematic of the flow loop experiments showing a straight and a stenosis model

A peristaltic pump (Ismatec, Germany) produced a steady flowrate of 100mL/min, in order to match the physiological Reynolds number, wall shear stress and wall shear rate as shown in figure 3. A fluid reservoir upstream of the pump provided fluid for circulation and a port for gasification with 5% CO_2 in air. Modified endothelial cell culture media, using 10% dextran (D4876, Sigma-Aldrich, Canada), to match blood viscosity (3.5cP), was perfused over the confluent EC layer for 12 and 24 hours. In the section upstream of the model, a length of straight tubing (Silicone peroxide cured, Masterflex, Canada) was used to obtain a parabolic velocity profile at the entrance of the straight and stenosed models inserted in the flow loop using connectors.

At the end of the perfusion, the models were disconnected from the flow loop; the media drained and the monolayer observed using inverted microscopy. Tubes were fixed with freshly made 2% paraformaldehyde in 1X PBS (BP399-1, Fisher Scientific Canada,)**.** Cells were stained for 60 seconds using crystal violet 4% solution (212525, BD Biosciences, Canada). A CCD camera and accompanying software installed on a Leica inverted microscope was used to obtain digital images of representative sections of the models for morphometric analysis.

Platelets: Venous blood (120cc) was obtained from the femoral vein of normal pigs in 30cc syringes containing 5cc of acid citrate dextrose. Platelet-rich plasma (PRP) was prepared by centrifugation of whole blood at 500*G* for 15 min. Platelets were then obtained by centrifugation of PRP at 800*G* for 10 min and resuspended in Hanks' balanced salt solution (HBSS)-HEPES buffer with 0.4 mM EDTA (HBSS-EDTA) (pH 6.5) free from Ca^{2+} and Mg^{2+}. After removal of red blood cells by a low centrifugation, the isolated platelets were resuspended in a HBSS-HEPES buffer (pH 7.4) with Ca^{2+} (1.3 mM $CaCl_2$) and Mg^{2+} (0.81 mM $MgSO_4$) and adjusted to a final concentration of 250×10^6cells/mL using an automated cell counter (T890, Beckman Coulter, Inc., Fullerton, CA). After washing, the platelet suspension was incubated with 300 µCi ^{51}Cr (Amersham International) for 40 minutes. The suspension was then centrifuged to remove unbound ^{51}Cr, and the radiolabeled platelets resuspended in plasma, with a viscosity of 1.2cP.

Simultaneous parallel pairwise perfusion of straight and stenosis models, coated for 4 hours using fibronectin (20µg/mL, F0895, Sigma-Aldrich, Canada) were performed. The flow within the models was adjusted to 100mL/min with a peristaltic pump (Masterflex, Cole-Parmer). The resulting fluid parameters are shown in figure 3. The models were placed in a thermoregulated plexiglass controlled water bath maintained at 37°C by an electric heating element. The platelet suspension was perfused for 15 min.

At the end of the experiments, the amount of platelets deposited on the models was quantified by counting the specific ^{51}Cr radioactivity associated with the models. The radioactivity was quantified, using a gamma counter (Minaxi 5000, Packard Instruments Co.). Platelet deposition (x 10^6/cm^2) on the models was calculated knowing the suspension platelet count (250 x 10^6) and the radioactivity in the models.

Results

Models: Assuming a fully developed flow at the entrance of the models, with a parabolic shaped velocity profile and using basic fluid dynamics, one can expect that the maximum shear stress observed on the proximal section of the stenosis to be about 2.8 times smaller than that observed in the stenosis throat based on the difference of diameter between those two sections as shown in equations 6 and 7.

$$\frac{A_{proximal}}{A_{throat}} = \frac{D_{proximal}^{\ 2}}{D_{throat}^{\ 2}} = 2 \Rightarrow D_{proximal} = 1.41 D_{throat} \quad (6)$$

$$\frac{\tau_{proximal}}{\tau_{throat}} = \frac{Q_{proximal} D_{throat}^{\ 3}}{Q_{throat} D_{proximal}^{\ 3}} = \frac{D_{throat}^{\ 3}}{(1.41 D_{throat})^3} = 2.8 \quad (7)$$

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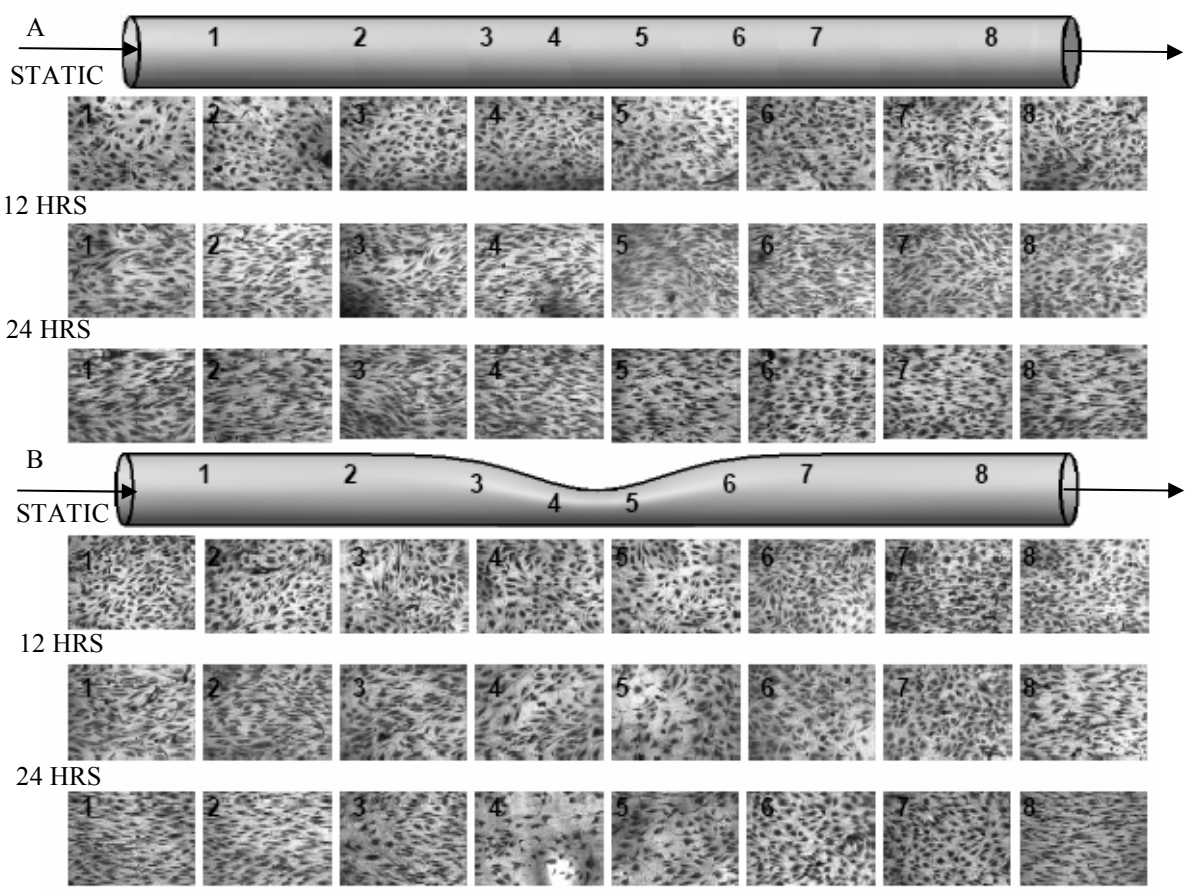

Figure 4: Endothelial Cell Morphology in Different Regions of the Asymmetric Stenosis Model
A) Straight Model, B) Stenosis Model

This means that, at the peak of the stenosis, for the perfusion endothelial cell experiments, the Reynolds number is 280, the shear rate 1560 s^{-1} and the shear stress 55 dyn/cm^2, and for the platelet perfusion, the Reynolds number is 798, the shear rate the same as for the EC experiments and the shear stress 19 dyn/cm^2, based on the assumption that the fluid behaves like a Newtonian fluid and that it obeys Poiseuille flow.

Endothelial Cells: To mimic a stenosis in an artery, endothelial cells were seeded onto Sylgard™ models and observed to investigate the effects of laminar and disturbed shear stress on the morphology of EC. These experiments were designed to allow the study of the response of endothelial cells to shear stress, in straight and stenosis models. In all cases cells remained healthy and attached to the Sylgard™ surface until the end of the experiments. Digital images were acquired in order to compare the different shear stress regions in the models.

Pictures illustrating the alignment of the cells in the different models, for the static controls and the different perfusion times are shown in figure 4. In static controls, for both geometries, cells showed a polygonal shape with no preferred alignment on the sides of the models. The perfusion of media in the models changed the cell morphology. Endothelial cells, which initially had the usual cobblestone shape, after 12 hrs of perfusion showed a preferential alignment, i.e. elongated shape, with the long axis parallel to the direction of the flow. This was found everywhere except in the stenosis region and the low shear stress recirculation zone after the stenosis. Cells at the peak of the stenosis were less dense than in other regions of the model. In the recirculation zone where the flow is disturbed, cells had a polygonal shape and were more confluent. There appears to be a zone, near the predicted reattachment point, where cells change morphology and instead of being randomly oriented became more aligned. After 24 hrs, most of the cells were aligned with the flow, except in the recirculation zone and at the stenosis peak. In these regions, the pattern was similar to the 12 hours models.

Platelets: The models were fixed and used to quantify the platelet adhesion to the model using a scintillation counter. For validation of the model, fibronectin coating on the models and pig blood have been used in a straight and stenosis models as a positive control. A two way ANOVA showed the overall adhesion in the two models to be significantly different ($P<0.05$).

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Discussion

The results of this study, show that in a realistic tri-dimensional stenosis model, that include both laminar and disturbed wall shear stress regions, endothelial cells showed distinctive morphology, i.e. aligned with the direction of flow in the laminar wall shear stress regions an randomly oriented cobblestones in the disturbed wall shear stress region. At the peak of the stenosis, cells seemed to have detached from the models, as these regions were less dense than the other regions. Platelets have adhered more readily, in greater proportions, in the stenosis models than in the straight models.

At the wall, the shear stress and shear rate are maximum. Both endothelial cells and platelets (concentrated at the wall due to the axial concentration of red blood cells), are exposed to the higher fluid shear stress. In this model, there is a large increase in shear stress at the peak of the stenosis and a drastic reduction in the low shear stress recirculation zone [22].

Shear stress induces cytoskeleton changes, both in laminar and disturbed shear stress. Most of the previous experiments were performed in simplified model, using either cone and plate viscometer [9,23] or parallel plates and a step to simulate the flow patterns in a stenosis [13,10,24]. Morphological studies on the alignment of cells throughout the vascular tree, especially in regions of bifurcations and curvatures were also made showing the same basic results [25].

The function of endothelial cells is also modified in various shear fields. Details on the functionality of the cells in our models still need to be quantified, as well as the exact dynamics at the reattachment point. In physiological conditions, different forces act in conjuction on the endothelial cells. The flow is pulsatile in nature, which makes the reattachment point unstable and creates regions of oscillatory shear stress. The simplistic models do not respect the physiological tri-dimensional nature of arteries. Our model, which can be modified as desired can be used to explain interactions between blood components and the endothelium as well as reactions between the endothelium and prosthetic devices, such as angioplasty balloons and stents.

As reported, for our preliminary results, using fibronectin coating and pig blood, adhesion of platelets was seen in positive controls. There is still insufficient data to comment on the interaction between the platelets and the endothelium in the models. The stenotic region creates a high shear stress region [4], known to cause the activation and aggregation platelets [26,6], and followed by a low shear stress recirculation region where platelets can get trapped and adhere more readily to the surface. In the literature, platelets are exposed to high shear stress, which may be non physiological (100 dyn/cm^2), for long periods of time, also not physiologic, as blood flow is pulsatile, resulting in fluctuating levels of shear stress and low retention time in those high shear stress regions. Also, most studies do not consider the shear stress and the shear rate separately. The conditions used in our experiments, are more realistic of what platelets may experience *in vivo* and can provide new insights on the mechanisms that occur between blood components, endothelium and hemodynamic forces.

Conclusion

This work provides a novel technique to study the effects of hemodynamic forces on the interaction between endothelial cells and platelets. We have demonstrated the ability to form a confluent monolayer of endothelial cells within the model. It has also been shown that platelets will attach to the model wall. In the future, this model will provide a better understanding of the mechanisms involved in atherosclerosis and of the influence of hemodynamics on coronary artery disease. Ultimately, this research will lead to better treatment and diagnosis of coronary artery disease.

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References

[1] AMERICAN HEART ASSOCIATION (2001): 'Heart disease and stroke statistics-2003 update',

[2] DEBAKEY, M.E.; LAWRIE, G.M. and GLAESER, D.H. (1985): Patterns of atherosclerosis and their surgical significance, *Ann Surg*, **201**, pp. 115-131

[3] YOUNG, D.F. and TSAI, F.Y. (1973): Flow characteristics in models of arterial stenoses. I. Steady flow, *J Biomech.*, **6**, pp. 395-410

[4] ZHANG, J.N.; BERGERON, A.L.; YU, Q., SUN, C.; MCINTIRE, L.V.; LOPEZ, J.A. and DONG, J.F. (2002): Platelet aggregation and activation under complex patterns of shear stress, *Thromb. Haemost.*, **88**, pp. 817-821

[5] BLUESTEIN, D.; NIU, L.; SCHOEPHOERSTER, R.T. and DEWANJEE, M.K. (1997): Fluid mechanics of arterial stenosis: relationship to the development of mural thrombus, *Ann. Biomed. Eng*, **25**, pp. 344-356

[6] KROLL, M.H.; HELLUMS, J.D.; MCINTIRE, L.V.; SCHAFER, A.I.; and MOAKE, J.L. (1996): Platelets and shear stress, *Blood*, **88**, pp. 1525-1541

[7] GIMBRONE, M.A.; JR., TOPPER, J.N.; NAGEL, T.; ANDERSON, K.R. and GARCIA-CARDENA, G. (2000): Endothelial dysfunction, hemodynamic forces, and atherogenesis, *Ann.N.Y.Acad.Sci.*, **902**, pp. 230-239

[8] NEREM, R.M., LEVESQUE, M.J. and CORNHILL, J.F. (1981): Vascular endothelial morphology as an indicator of the pattern of blood flow, *J. Biomech. Eng*, **103**, pp. 172-176

[9] BLACKMAN, B.R.; GARCIA-CARDENA, G. and GIMBRONE, M.A., JR. (2002): 'A new in vitro model to evaluate differential responses of endothelial cells to simulated arterial shear stress waveforms', *J.Biomech.Eng*, **124**, pp. 397-407

[10] TARDY, Y.; RESNICK, N.; NAGEL, T.; GIMBRONE, M.A., JR. and DEWEY, C.F., JR. (1997): Shear stress gradients remodel endothelial monolayers in vitro via a cell proliferation-migration-loss cycle, *Arterioscler. Thromb. Vasc.Biol.*, **17**, pp. 3102-3106

[11] IBA, T. and SUMPIO, B.E. (1991): 'Morphological response of human endothelial cells subjected to cyclic strain in vitro', *Microvasc.Res.*, **42**, pp. 245-254

[12] QIU, Y. and TARBELL, J.M. (2000): 'Interaction between wall shear stress and circumferential strain affects endothelial cell biochemical production', *J.Vasc.Res.*, **37**, pp. 147-157

[13] NAGEL, T.; RESNICK, N.; DEWEY, C.F. JR., and GIMBRONE, M.A., JR. (1999): 'Vascular endothelial cells respond to spatial gradients in fluid shear stress by enhanced activation of transcription factors', *Arterioscler.Thromb.Vasc.Biol.*, **19**, pp. 1825-1834

[14] GARCIA-CARDENA, G.; COMANDER, J., ANDERSON, K.R.; BLACKMAN, B.R., and GIMBRONE, M.A., JR. (2001): 'Biomechanical activation of vascular endothelium as a determinant of its functional phenotype', *Proc.Natl.Acad.Sci.U.S.A*, **98**, pp. 4478-4485

[15] RUGGERI, Z.M. (2003): 'Von Willebrand factor, platelets and endothelial cell interactions', *J.Thromb.Haemost.*, **1**, pp. 1335-1342

[16] HUO, Y. and LEY, K.F. (2004): 'Role of platelets in the development of atherosclerosis', *Trends Cardiovasc.Med.*, **14**, pp. 18-22

[17] BAUMGARTNER, H.R. and HAUDENSCHILD, C. (1972): 'Adhesion of platelets to subendothelium', *Annals Of The New York Academy Of Sciences*, **201**, pp. 22-36

[18] EINAV, S. and BLUESTEIN, D. (2004): 'Dynamics of blood flow and platelet transport in pathological vessels', *Ann.N.Y.Acad.Sci.*, **1015**, pp. 351-366

[19] BRUNETTE, J. (2005): 'Investigation Expérimenetal des contraintes hémodynamiques d'un fantôme d'artère sténosée',

[20] COMOLET, R. (1984): 'Biomécanique circulatoire',

[21] SIEBES, M.; CAMPBELL, C.S., and D'ARGENIO, D.Z. (1996): 'Fluid dynamics of a partially collapsible stenosis in a flow model of the coronary circulation', *J Biomech.Eng*, **118**, pp. 489-497

[22] DVINSKY, A.S.; and OJHA, M. (1994): 'Simulation of three-dimensional pulsatile flow through an asymmetric stenosis', *Med.Biol.Eng Comput.*, **32**, pp. 138-142

[23] DAI, G.; KAAZEMPUR-MOFRAD, M.R.; NATARAJAN, S.; ZHANG, Y.; VAUGHN, S.; BLACKMAN, B.R.; KAMM, R.D.; GARCIA-CARDENA, G.; and GIMBRONE, M.A. JR. (2004): 'Distinct endothelial phenotypes evoked by arterial waveforms derived from atherosclerosis-susceptible and -resistant regions of human vasculature', *Proc.Natl.Acad.Sci.U.S.A*, **101**, pp. 14871-14876

[24] GALBRAITH, C.G.; SKALAK, R. and CHIEN,S. (1998): 'Shear stress induces spatial reorganization of the endothelial cell cytoskeleton', *Cell Motil.Cytoskeleton*, **40**, pp. 317-330

[25] REIDY, M.A. and LANGILLE, B.L. (1980): 'The effect of local blood flow patterns on endothelial cell morphology', *Exp. Mol. Pathol.*, **32**, pp. 276-289

[26] CHUNG, I. and LIP, G.Y. (2003): 'Virchow's triad revisited: blood constituents', *Pathophysiol.Haemost.Thromb.*, **33**, pp. 449-454

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