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Microfluidic technology as an emerging clinical tool to evaluate thrombosis and hemostasis

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Abstract

Assessment of platelet function and coagulation under flow conditions can augment traditional static assays used to evaluate patients with suspected hemostatic or thrombotic disorders. Among the available flow-based assays, microfluidic devices require the smallest blood volume and provide multiple output options. These assays are based on the presence of wall shear stress that mimics *in vivo* interactions between blood components and vessel walls. Microfluidic devices can generate essential information regarding homeostatic regulation of platelet activation and subsequent engagement of the coagulation cascade leading to fibrin deposition and clot formation. Emerging data suggest that microfluidic assays may also reveal consistent patterns of hemostatic or thrombotic pathology, and could aid in assessing and monitoring patient-specific effects of coagulation-modifying therapies.

Introduction

Normal hemostasis involves a combination of cellular, soluble, and structural factors interacting in a coordinated fashion at vascular injury sites to stem blood loss. Alterations in the normal regulation of this process, whether in the setting of pro-thrombotic or hemorrhagic states, contribute to significant clinical pathology. Currently, assessment of patients with disorders of hemostasis involves a multifaceted evaluation of the various components of clot formation including coagulation factors, platelets, and ancillary thrombotic and lytic proteins. Activity assays for specific coagulation factors and adjuvant proteins, as well as various platelet function tests, are available to evaluate components

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separately. In an effort to measure many or all of the components of the hemostatic system in concert, “global assays” have been developed such as calibrated automated thrombogram (CAT) and thromboelastography (TEG) [1]. Rational use of these assays can provide a fairly thorough representation of a patient’s hemostatic status. However, the static nature of most of these assays neglects the effect of blood flow and the contribution of the endothelium to hemostasis. On the one hand, platelet adhesion and aggregation and von Willebrand factor (VWF) activity are shear stress-dependent [2], while on the other hand, coagulation, fibrin formation, and fibrinolysis are shear rate dependent [3]. Additionally, endothelial cells promote and inhibit clot formation by many mechanisms including secretion of soluble agents (VWF, Prostacyclin, ADPases) and surface-bound molecules (thrombomodulin, P- and E-selectin). Therefore, microfluidic assays are an alternative that may provide a more complete evaluation of hemostasis.

Historical Perspectives on flow-based assays

The combined efforts of many researchers led to the development of parallel-plate [4] and annular assays [5] over 40 years ago [6], complemented the existing cone-plate viscometers [7], and allowed for assessment of the interactions of blood cells and vessel wall components under physiologic flow conditions. This new field took shape in the early 1960’s with the development of an annular perfusion chamber, also known as the “Baumgartner chamber” to evaluate platelet-vessel wall interactions. Further refinement of the physical, chemical, and pharmacologic factors that influenced platelet and coagulation-related thrombosis led to the design of the parallel-plate, also known as the “Sakariassen chamber.”

The 1970’s and 1980’s witnessed a surge of flow devices that were instrumental to the evaluation of hemostatic function under flow. This concept was used for the evaluation of several disease states such as von Willebrand disease (VWD) [8–10], hemophilia [11], platelet storage pool defects [8], platelet receptor defects [12,13], and uremia [14]. In addition, the function of transfused platelets was also studied in flow-based assays [15]. Later studies focused on platelet rolling, adhesion, and aggregation on immobilized ligands and provided critical insights into shear-dependent receptor-ligand interactions [16–18]. Despite the wealth of knowledge in the basic physiology of hemostasis revealed by these groundbreaking studies, these early devices were never translated into clinical assays, in part because of the disadvantageous combination of relatively large blood volume requirements and low throughput.

Microfluidics

Microfluidic technology addresses some of the limitations of larger flow chambers by offering disposable standardized devices that allow for the reproducible analysis of hemostatic function under a wide range of shear stresses with low blood volume requirements. To date, this technology has been primarily used in the basic science setting to study the interactions between receptor-ligand binding, the effect of these interactions on platelet signaling pathways, and the role of hemodynamics; often in conjunction with assays evaluating coagulation [17,18]. Multiple options for the patterning of prothrombotic substrates and proteins on microfluidic devices provide the versatility needed for accurate simulations of vascular injury, allowing for further elucidation of normal hemostatic

pathways [19–21]. Furthermore, strategic arrangement of membrane-based devices to control flow of soluble pro- or anti-hemostatic factors has advanced our knowledge of the role of these factors in normal hemostasis and thrombus formation. For example, this approach led to the demonstration that both flux-dependent thrombin concentration and wall shear rate regulate fibrin polymerization and deposition under flow [3]. A similar method was used to show that ADP-induced platelet aggregation under flow is directly proportional to the agonist flux [22].

Many standardized *in vitro* assays have been developed for the purpose of analyzing platelet-related hemostatic and thrombotic properties at baseline compared to various disease-related or treatment-induced conditions. These include platelet aggregometry, flow cytometric evaluation of surface activation markers, scanning electron microscopic evaluation of platelet spreading, clot retraction, and western blot analysis of platelet signaling protein phosphorylation. The physiologic relevance of many of these assays may be called into question as their static nature does not account for certain critical interactions, such as that of GP1bα and VWF, which is significantly relevant under shear stress. One may argue that biologically representative assays must include a component of flow, since disorders of bleeding and thrombosis are modulated in many cases by flow-dependent mechanisms.

Currently, the majority of clinical hemostatic analyses are static clotting-based assays sensitive to severe factor deficiencies or inhibition. Available assays that incorporate some aspect of shear stress include the Platelet Function Analyzer 100 (PFA-100®), VerifyNow®, and the Multiplate® analyzer. Each of these offers a specific, but likely incomplete, picture of hemostasis, prompting a desire for an integrated test of global hemostasis [23,24]. In addition, the majority of these assays require relatively large amounts of blood; some laboratories require as much as 4 mL of whole blood to isolate plasma for specific coagulation or platelet function assays. In comparison, the low blood volume requirement (as little as 50 μL) of a microfluidic system is advantageous in situations of limited blood supply, such as premature infants at risk for anemia or individuals with poor venous access who could more easily provide a small-volume fingerstick or heelstick sample.

There are many excellent recent reviews on the subject of the design, construction, and use of microfluidic chambers in the setting of basic science studies of coagulation [23,25,26]. For example, microfluidic technology has been used as a model to define novel findings in platelet biology such as shear-dependent platelet aggregation [27,28] and platelet-VWF bond interactions [29]. The use of microfluidic technology has also contributed significantly to VWF biology such as in further characterization of stimulated release of ultra-large VWF (ULVWF) from endothelial cells that form “VWF strings” suggesting that flow rates dramatically affects the conformation of VWF [30]. Microfluidic technology has also been useful in the functional study of ADAMTS13 (A Disintegrin And Metalloprotease with ThromboSpondin type 1 repeats, member 13) which cleaves VWF [31] and modulates its prothrombotic activity. This review provides a summary of the recent contributions of microfluidics for clinical applications in hemostasis and thrombosis, and focuses on the unique opportunities offered by microfluidic devices to add a flow-based component to current hemostatic evaluations in patients with bleeding and thrombotic disorders.

Current Applications for the Use of Microfluidics in Clinical Assays

Three main current applications of microfluidic-based flow assays include (1) quantifying the normal range of responses within the healthy population, (2) evaluating hemostasis in various disease states, and (3) evaluating the effect or dose titration of platelet- or coagulation-modifying drugs that may be used by patients with bleeding or thrombotic disorders.

Quantifying the normal range of response in flow assays

Minor clinical bleeding is relatively common in the general population, ranging from increased bruising and epistaxis to increased blood loss following surgeries, dental procedures, and childbirth. Microfluidic assays have the potential to advance our knowledge of the normal contributions of multiple interacting factors into the overall clinical bleeding phenotype. In the largest flow assay study to date (n=104 individuals), microfluidic devices were used to identity sources of variability in platelet adhesion and aggregation on type I collagen under flow [32]. The results showed that plasma VWF levels and glycoprotein 6 gene (*GP6*) genotypes imparted the greatest influence on platelet accumulation. One recent and more comprehensive analysis of the signaling contributions made by individual platelet receptors systematically evaluated 52 adhesive surfaces (fabricated with a combination of ligands for nine different platelet receptors) and used eight measured parameters and hierarchical clustering to determine the relative contribution of these receptors to platelet aggregate growth and thus “phenotype” platelet adhesion and aggregation [33]. This combinatorial approach, partially made possible by the high-throughput capability of microfluidics, led to the discovery of unique signatures of genetic platelet defects.

Evaluating hemostasis in various disease states

A challenge in diagnosing and treating bleeding disorders is predicting bleeding risk, which does not always correlate with coagulation factor levels. For example, two individuals with hemophilia A and similar factor VIII (FVIII) levels may experience different clinical bleeding phenotypes [34,35]. It is possible that global assays, such as whole blood flow assays that integrate the entire hemostatic system, could provide better predictions of bleeding severity than factor levels.

Microfluidic flow assays recapitulate some salient features of bleeding disorders. For example, abnormal thrombus formation is observed with blood from FVIII-null mice at venous shear stresses but not at arterial shear stresses, in agreement with the presentation of bleeds in low shear stress environments (joints, muscles) in humans [36]. Microfluidic assays have also been used to measure fibrin deposition and platelet aggregate size in patients with hemophilia [37,38]. While fibrin deposition was clearly different between severe and mild hemophilia samples, the system could not discriminate well between severe and moderate FVIII deficiency. Interestingly, it was observed that platelet aggregate size, but not fibrin deposition, could discriminate severe and moderate FVIII deficiencies [38].

Flow assays have also been used to evaluate VWD, such as demonstrated in the study by Sugimoto et al. that allowed for the discrimination of VWD types 2A and 2B in whole blood

[39] and the recent report of novel mutations in the A1 domain that mediate abnormal VWF-platelet binding in VWD type 2A [29]. As compared to the static nature of current collagen binding assays [40], microfluidic assays may allow for the characterization of abnormalities in VWF-collagen binding under physiologic shear stresses and can further elucidate novel pathology in this relationship. Future microfluidic-based approaches to VWD, especially those with type 1 disease, could involve comparison of individual patients' aggregation patterns to those of existing cohorts, once established, to help predict clinical bleeding.

On the other end of the hemostatic spectrum, patients who suffer vascular thromboembolic events are treated with anticoagulant (venous) or antiplatelet (arterial) therapies. A common clinical problem is the determination of appropriate length of therapy when faced with a paucity of data about coagulation status. Assays such as D-dimer levels or thrombin-antithrombin (TAT) complexes can be misleading as they can be affected by various changes in homeostasis (inflammation, infection, recent exercise, physical or mental stress, etc.). Real-time evaluation of the thrombotic potential of a patient's status would ostensibly be very helpful to determine an appropriate time to stop anticoagulation/antiplatelet therapy. Perhaps such an evaluation could soon be possible with recent advances in microfluidic assays that have provided the ability to investigate pathological shear stresses in channels that mimic stenotic vessels [41] or those with resultant turbulent flow. Similarly, microfluidic assays have been used to evaluate the spatial distribution of tissue factor on coagulation, which may have bearing on treatment of sepsis-related disseminated intravascular coagulation or venous thromboembolism [42]. Another promising trend is the use of microfluidics to evaluate patient-specific thrombotic potential and response to specific pro-coagulant agents such as chemotherapy [43].

Monitoring alterations in normal hemostasis and response to therapy

Microfluidic assays may have the potential to guide personalized dosing of antiplatelet agents. For example, microfluidic assays are sensitive to COX-1, $P2Y_1$ and $P2Y_{12}$ inhibitors and can detect differences in inter-individual sensitivity to aspirin [44] or inhibitors of the ADP receptors ($P2Y_1$ and $P2Y_{12}$) [45] though not to apyrase or a $P2X_1$ inhibitor [46]. Dose-response curves in an occlusive microfluidic device that models thrombotic occlusion determined increased efficacy for eptifibatide compared to aspirin [47]. Moreover, microfluidics have proven useful in the evaluation of novel antiplatelet agents and allow for faithful comparisons to existing agents [48].

Promising results have also been reported with evaluation of anticoagulants in microfluidic devices. Dose-response curves have been measured on collagen-TF surfaces at venous and arterial shear rates with heparin, argatroban, abciximab, and OS-1 (GP1bα antagonist) and at drug concentration sensitivity greater than that available through TEG [49]. Microfluidic assays have also been used to evaluate patient-specific thrombotic potential and response to anticoagulation in a novel point of care coagulation assay device [50]. The disposable microfluidic device used in this study uses a fluorescence-based anti-Factor Xa assay to monitor the overall anti-thrombotic effect of unfractionated heparin, tinzaparin, and enoxaparin with high reliability (average coefficients of variation <10% and R^2>0.98). This approach avoids the pitfalls of the standard point of care devices that rely on clot-based

endpoints with high variability and low reliability, in addition to measuring only certain portions of the coagulation pathway. Similarly, a microfluidic assay was used to study thrombus formation on collagen and von Willebrand factor matrices, in addition to a dose-response effect of abciximab, demonstrating the ability to obtain a large number of data points per single patient sample using small blood volumes and high throughput approach [51]. Similarly, a recent report describes the development of a low-cost paper-based microfluidic system to help titrate oral anticoagulation doses [52]. In addition to measuring a patient's anticoagulation status, microfluidic technology has also allowed for the study of the response to replacement or bypass therapies in individuals with FVIII deficiencies [38] demonstrating that replacement (FVIII) and bypass therapy (rFVIIa) can result in significant increases in fibrin formation that mirror the clinical hemostatic response.

Limitations to widespread clinical use of microfluidic-based technologies

While microfluidic-based technology has the potential to significantly advance our understanding of basic hemostasis and offers numerous potential clinical applications, some significant drawbacks remain. Complex designs allow for strategic utilization of multiple shear stresses and input types, but may be cumbersome for widespread clinical use [53,54]. Another potential disadvantage of using microfluidic systems is the labor-intensive device-patterning and intricate setup, though several self-contained commercial systems are now available that are quite straightforward to operate, with reproducible patterning strategies that decrease device variability. While the basic materials used to create microfluidic devices are relatively economical, a major limitation is the current expense of purchase and upkeep of the essential sophisticated image capture and analysis apparatus, such as confocal microscopy and real-time imaging acquisition software. Currently, these pose significant logistical roadblocks to the clinical use of microfluidic technology and further research into translational approaches will likely lead to advancements in bringing this technology to the clinical area.

A major hurdle impeding clinical adoption of these devices is the lack of well-established normal results. This is primarily due to the relative novelty of the technology as well as to the inter-assay variability due to lab-specific differences in the specifics of flow chamber construction and patterning as well as the source, composition, and concentration of prothrombotic substrate. While some publications have begun to address this issue [32], further investigation is needed to more thoroughly categorize normal variation prior to mainstream clinical usage. In an attempt to standardize microfluidic strategies, The International Society of Thrombosis and Hemostasis has advocated for standardization in the measurement of thrombus formation in flow chamber-based assays via their Biorheology Subcommittee [55–58].The group has suggested comparative studies to determine optimal assay conditions (chamber type, surface coating, pre-analytical blood collection and storage, image recording, and image quantification), as well as a cost-efficacy comparison evaluating frequently used custom-made and commercial devices. A combined effort by the scientific community is encouraged to continue this necessary standardization.

Future Directions

The future of microfluidic assays likely will include endothelialized microvasculature models [30,59,60] and microfluidic models of vessel stenosis [27,28,61,62]. Microfluidic assays that incorporate endothelial cells can model microvascular thrombosis in diseases such as sickle cell disease and hemolytic uremic syndrome, offering some insight into the versatility and clinical relevance of the microfluidic model as a biophysical assay of hemostatic pathophysiology [60]. This particular study demonstrated that hydroxyurea quantitatively decreases sickle cell-related microvascular obstruction and that the platelet aggregation-decreasing effects of eptifibatide are increased under shear stress, thus potentially increasing its utility in hemolytic uremic syndrome.

One significant advantage of such microvasculature model design is the ability to create circular, rather than rectangular, vessel geometry [30]. This approach minimizes the variations in shear stress across the channel, thus more closely replicating blood vessels. However, the constant flow rate/shear stress used in many microfluidic assays is dissimilar to the pulsatile flow rates experienced *in vivo* and standardization of flow rates and pulses will be necessary for further development of the field. Another advantage of microvasculature models is the incorporation of endothelial mediated pro- and antithrombotic mechanisms, such as the release of VWF from damaged or activated endothelial cells [30]. The flexibility of microfluidic technology allows for the design of assays that can more appropriately model the various diameters and shear stresses of various arteries, veins, and even micro-capillaries [60]; this allows for the efficient evaluation of disease process over a variety of vasculature-specific models. Advancements in engineering, such as microscafffold structures [63] or nanosensing technologies [64] may allow for novel detection of hemostatic biomarkers that can further differentiate primary and secondary hemostasis. The advancement of these novel output technologies may obviate the need for advanced image capturing and processing software and allow for an easier translation of microfluidic technology to the clinical realm.

The next step in microfluidic research will be to bring these assays from the basic science research bench into the clinical lab and eventually the bedside. In the setting of trauma, which is often associated with acute coagulopathy, a simplified bedside microfluidic assay could provide real-time guidance of blood product replacement in a manner similar to that proposed for TEG [65,66] but with additional analysis of platelet function. Additionally, the application of microfluidics to global hemostasis assessment in the setting of bleeding phenotypes has the potential to determine appropriate factor replacement doses to target appropriate fibrin/thrombin formation without pro-thrombotic overcompensation.

A cooperative approach between engineers and clinicians could usher in an era of flow-based global hemostasis evaluation allowing for guided blood product administration to correct acute coagulopathy of trauma, gauge clinical bleeding risk to guide factor replacement or bypass therapy in patients with confirmed/suspected bleeding disorders, and evaluate thrombotic potential in patients with history of, or risk for, thromboembolic disease to guide anticoagulant choice and dose. Overall, microfluidic technologies offer a unique combination of physiologic accuracy and small blood volume requirements to evaluate a

large number of patient-specific factors that has the potential to individualize therapeutic options for disorders of thrombosis or hemostasis.

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Microfluidic Assay Highlights

- Microfluidic technologies have been evolving over the last sixty years
- Microfluidic devices allow platelet and coagulation analysis under flow conditions
- Other advantages include low blood volume requirements and multiple output options
- Microfluidic assays can aid in monitoring patient-specific drug effects and dosing
- Continued adaptations are needed to optimize these assays for routine clinical use

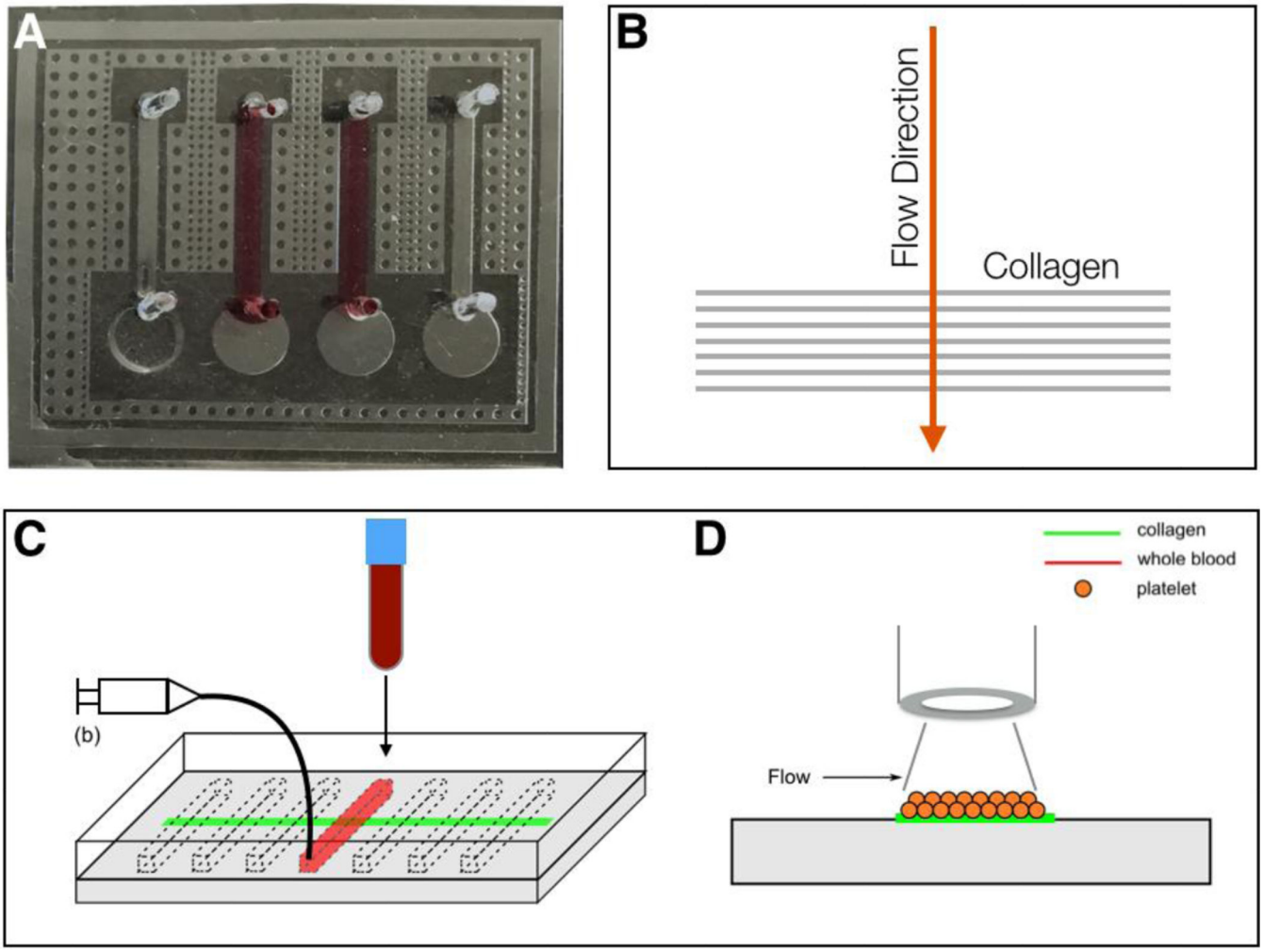

Figure 1.

Representative Image of a Microfluidic Device and Schematic Diagrams to Demonstrate the Assay. A) A representative image of a PDMS microfluidic device patterned with four channels, two filled with a red dye. B) A schematic example of a surface that demonstrates left to right collagen patterning with perpendicular flow. Other devices may use small circular patterns of substrates. C) Whole blood is perfused onto a chamber connected to a syringe pump generating desired shear rate. D) A cross sectional appearance of a channel, whereby platelet deposition can be detected with bright field microscopy.

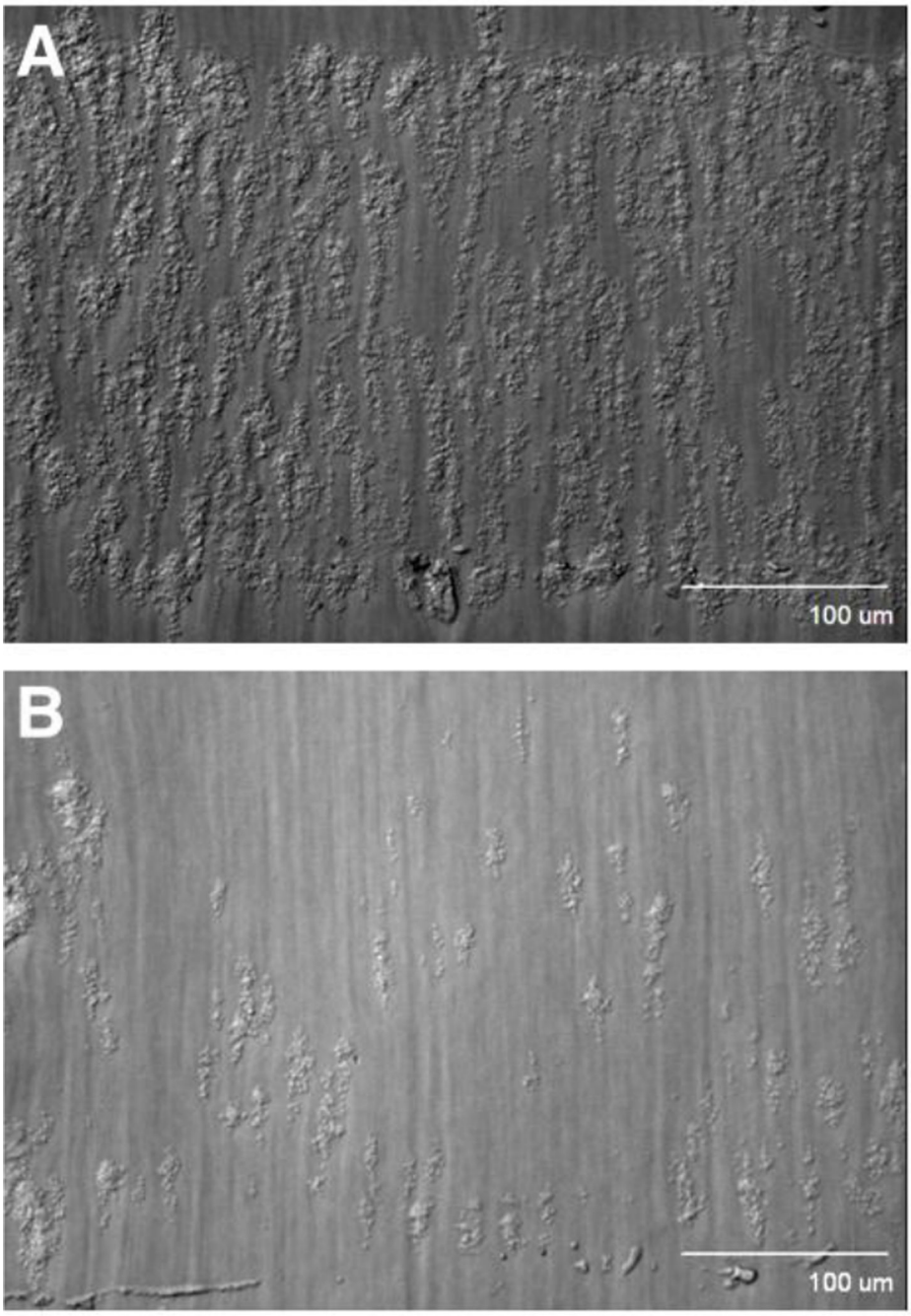

Figure 2.

Formation of platelet aggregates under flow conditions from citrated whole blood. Re-calcified whole blood is perfused at a consistent flow rate across a collagen strip. Platelets bind to collagen through collagen receptors and through VWF mediated adhesion in the direction of blood flow. The degree of platelet adhesion/aggregation is related to the plasma concentration of VWF A) Representative image of platelet adhesion seen with a healthy

control. B) Representative image of decreased platelet adhesion see in a patient with Type 1 Von Willebrand Disease

Table 1

Recent relevant studies using microfluidic assays for studying hemostasis.

Author	Journal	Year	Title	Conclusions
Muthard RW, et al	ATVB	2015	Fibrin, fibrinogen, and transclot pressure gradient control hemostatic clot growth during human blood flow over a collagen/tissue factor wound	Hemostatic clotting is dependent on core-localized thrombin that triggers platelet p-selectin expression and is highly regulated by fibrin and transclot pressure. Fibrinogen plays a role in venous but not arterial conditions.
Zhu S, et al	Thromb Res	2014	Contact activation of blood coagulation on a defined kaolin/collagen surface in a microfluidic assay	Alteration in flow conditions can affect the activity of the contact pathway, which is less efficient in prompting thrombin generation compared to the extrinsic pathway.
Onasonga-Jarvis AA, et al	J Thromb Haemost	2014	Thrombin generation and fibrin formation under flow on biomimetic tissue factor-rich surfaces	Fibrin deposition requires perturbations in the flow field that protect reactions from dilution by flow under venous and arterial conditions. FVIII and FIX have a modest effect on fibrin deposition at high tissue factor (TF) concentrations but are necessary for fibrin deposition at low TF concentrations. FXI amplifies thrombin generation under flow at both low and high TF concentrations.
Colace T, et al	J Thromb Haemost	2014	Microfluidic Assay of Hemophilia Blood Clotting: Distinct Deficits in Platelet and Fibrin Deposition at Low Factor Levels	Hemophilia patients with <1% factor activity exhibited 50% reduction in platelet deposition. Defects in fibrin deposition were seen below 13% factor activity.
De Witt S, et al	Nat Comm	2014	Identification of Platelet Function Defects by Multi-Parameter Assessment of Thrombus Formation	Description of platelet adhesion on 52 different surfaces with 8 output paremeters describing different stages of thrombus formation, demonstrating the diagnostic utility of *in vitro* flow- based approaches to suspected disorders of hemostasis or thrombosis
Li M, et al	PloS one	2014	Microfluidic Thrombosis Under Multiple Shear Rates and Antiplatelet Therapy Doses	Dose-response curves for heparin, eptifibatide, and ASA were dependent on shear rate, which varied inversely with thrombus stability
Li R, Diamond S	Thromb Res	2014	Detection of Platelet Sensitivity to Inhibitors of COX-1, $P2Y_1$, and $P2Y_{12}$ Using a Whole Blood Microfluidic Flow Assay	Receiver-Operator Characteristic curve R-values can serve as a self-normalized metric of platelet function for a single blood sample. Aggregation was increasingly inhibited by antagonists of COX-1<$P2Y_{12}$<$P2Y_1$<Combo $P2Y_{1+12}$
Neeves, et al	PloS one	2013	Sources of Variability in Platelet Accumulation on Type 1 Fibrillar Collagen in Microfluidic Flow Assays	VWF levels were positively correlated to V_{PLT} and SC at wall shear rates, and were thereby the strongest determinant of platelet accumulation. Individuals with Ag genotype of P6 gene had lower platelet accumulation compared to individuals with AA genotype. Citrate appears to irreversibly diminish platelet function.
Hosokawa K, et al	Thromb & Haemost	2013	Analysing Responses to Aspirin and Clopidogrel by Measuring Platelet Thrombus Formation Under Arterial Flow Conditions	ASA and thienopyridine markedly reduced growth and stability of platelet thrombi. Platelet thrombogenicity in ASA-treated patients is associated with either coallagen-induced aggregation or circulating platelet-monocyte aggregates, but in those receiving dual antiplatelet therapy, it depends more on ADP-induced aggregation.
Onasoga-Jarvis A, et al	PloS one	2013	The Effect of FVIII Deficiencies and Replacement/Bypass Therapies on Thrombus Formation Under Venous Flow Conditions in Microfluidic and Computational Models	No difference in fibrin formation was seen between severe and moderate hemophilia A, though platelet aggregate size was significantly larger for moderate factor VIII deficiency. In moderate deficiency local thrombin concentration is high enough to induce platelet activation, but too low to support fibrin formation. Platelet adhesion is needed for fibrin formation. Individuals treated with bypass therapy (rFVIIa) had a reduced lag time in fibrin accumulation compared to healthy controls, and experienced changes in fibrin

Author	Journal	Year	Title	Conclusions
				dynamics that could lead to a prothrombotic state, a result not seen in those treated with rFVIII.
Van Kruchten, et al	Platelets	2012	Measurement of Whole Blood Thrombus Formation Using Parallel-Plate Flow Chambers – A Practical Guide	A practical guide for use of parallel-plate flow chambers, addressing surface coating, blood flow and shear rate calculations, pre-analytical variables, and analysis procedures
Li M, et al	Lab on a Chip	2012	Microfluidic System for Simultaneous Optical Measurement of Platelet Aggregation oat Multiple Shear Rates in Whole Blood	Description of design, fabrication, testing, and application of a new microfluidic device for measurement of the entire process of platelet thrombosis in whole, unlabeled blood at multiple shear rates

Abbreviations: ADP, adenosine diphosphate; ASA, acetyl-salicylic acid; rFVII, recombinant factor VII; rFVIIa activated recombinant acstudying hemostaitor VII; SC, platelet surface coverage; V_{PLT}, platelet accumulation; VWF, von Willebrand Factor