In Vitro and *Ex Vivo* Approaches to Evaluate Next-Generation Tobacco and Non-Tobacco Products on Human Blood Platelets

Sherry L. Spinelli,¹ Katie L. Lannan,² Shannon G. Loelius,³ and Richard P. Phipps¹⁻³

Abstract

Human blood platelets are major hemostatic regulators in the circulation and important in the mediation of chronic inflammation and immunomodulation. They are key elements that promote cardiovascular pathogenesis that leads to atherosclerosis, thrombosis, myocardial infarction, and stroke. New information on tobacco use and platelet dysregulation shows that these highly understudied vascular cells are dysregulated by tobacco smoke. Thus, platelet function studies should be an important consideration for the evaluation of existing and next-generation tobacco and non-tobacco products. Novel *in vitro* approaches are being sought to investigate these products and their influence on platelet function. Platelets are ideally suited for product assessment, as robust and novel *in vitro* translational methods are available to assess platelet function. Furthermore, the use of human biological systems has the advantage that risk predictions will better reflect the human condition.

Keywords: cardiovascular disease, *in vitro* testing, inflammation, nicotine, platelet, tobacco

Introduction

THE RISK OF DEVELOPING chronic inflammation and subsequent cardiovascular disease (CVD) is greatly increased by prolonged exposure to tobacco products, such as the smoke inhaled from cigarettes, hookah, cigars, and pipes.¹ Human blood platelets are an important cell type adversely affected by tobacco consumption.² Platelets are key mediators of chronic inflammation and drive the development of cardiovascular sequelae that lead to thrombosis, myocardial infarction (MI), and stroke.³⁻⁵ Despite the fact that platelets are dysregulated by tobacco use and are central players in these processes, they are not a first consideration for toxicology testing and safety assessment of new tobacco and tobacco-free products.

In 2009, the US Food and Drug Administration (FDA) was given authority by the “Family Smoking Prevention and Tobacco Control Act” to regulate the marketing and composition of tobacco products to reduce harm and improve public health.⁶ Abstinence from tobacco product use is strongly recommended, and programs to encourage and aid individuals in ceasing to use these products have had some success. However, not all individuals are able or willing to cease

their product usage. With the advent of tobacco harm reduction strategies, modified risk products are in development and in the marketplace; however, these products require extensive research to evaluate their content, toxicity potential, and to provide information on the safety and quality of the product to the consumer.

Alternative approaches for traditional toxicology testing and safety assessment are being championed worldwide to better understand disease mechanisms associated with tobacco and alternative product use, and to design and validate novel *in vitro* testing for new and modified products. Mechanistic human-based dynamic models that mimic complex biological systems are replacing traditionally used animal model systems. Animal systems sometimes poorly predict human responses to environmental and chemical toxicants, and only sometimes provide information regarding the underlying mechanisms associated with disease.

While development of *in vitro*-based toxicology testing has been challenging, a variety of new models and strategies that better reflect human biology and improve outcome predictions are becoming more reliable and accepted.^{7,8} Complex *in vitro* tissue and organ models have been established that include assessment of cardiovascular and vascular systems.⁹

Departments of ¹Pathology and Laboratory Medicine, ²Environmental Medicine, and ³Microbiology and Immunology, University of Rochester School of Medicine and Dentistry, Rochester, New York.

© Sherry L. Spinelli et al., 2017; Published by Mary Ann Liebert, Inc. This Open Access article is distributed under the terms of the Creative Commons Attribution Noncommercial License (<http://creativecommons.org/licenses/by-nc/4.0/>), which permits any noncommercial use, distribution, and reproduction in any medium, provided the original authors and the source are credited.

This article reviews the utility and novel application of *in vitro* systems for the evaluation of current and next-generation tobacco products on human blood platelet in the context of inflammation and CVD risk.

The Complex Life of the Platelet

The role of platelets is often underestimated and thought to be mainly associated with wound healing and blood clotting. Platelets are increasingly recognized as key elements in inflammation and CVD, contributing to numerous pathophysiologic conditions when inappropriately activated.¹⁰ Platelets are the second most numerous cells in the circulation ($2\text{--}4 \times 10^8/\text{mL}$) and are produced by a progenitor bone marrow cell, the megakaryocyte.¹¹

Despite lacking a nucleus, platelets have sophisticated mechanisms to regulate gene expression and respond to stimuli. They have organelles (e.g., mitochondria, Golgi apparatus, granules) and contain alpha, dense, and lysosomal granules.¹² Dense granules contain many prothrombotic mediators that aid in autocrine and paracrine signaling to amplify the platelet activation response. Alpha granules contain a wide repertoire of molecules, including adhesion receptors, immune molecules, and proinflammatory cytokines, to name a few.

Platelets are also capable of synthesizing prostaglandins, including thromboxane A_2 (TXA₂) from arachidonic acid on stimulation. Notably, platelets have a diverse transcriptome,¹³ undergo signal-dependent protein translation,^{14,15} and express transcription factors, such as nuclear factor kappa B (NF κ B)^{16–20} and peroxisome proliferator-activated receptor gamma,^{21,22} both of which have nongenomic roles.²³

Platelets also secrete membrane vesicles collectively termed, “extracellular vesicles” (EVs), which are a heterogeneous group of small transcellular regulators (e.g., microparticles [MPs], exosomes, oncosomes) that are released by many cell types.²⁴ EV cargo composition and function are determined by many factors, including their cell of origin, differential expression in response to disease, and environmental stressors. Platelets are highly enriched with small regulatory RNAs, microRNA (miRNA), which are abundantly released in EVs into the circulation.²⁵ All of these features afford platelets a unique versatility to rapidly respond to vascular stimuli and efficiently regulate hemostasis.

While platelets are critical in maintaining hemostasis when injuries are sustained or insults are present, platelet dysregulation can result in excessive inflammation and thrombosis, or bleeding diatheses (Fig. 1).^{26,27} Platelets function as circulatory sentinels that maintain hemostasis by (1) sensing insult or injury, (2) creating a physical barrier, (3) secreting alpha and dense granules containing proinflammatory mediators that activate and recruit additional platelets and leukocytes, and (4) activating endothelial cells. Endothelial cells lining the vascular wall and leukocytes are signaled by platelet factors, and the ensuing cross talk can lead to amplification of dysregulation and the development of disease pathology.^{28,29}

The platelet: quintessential Jekyll and Hyde

Under physiological conditions, platelets are kept in an inactive state through release of nitric oxide and prostaglandin I_2 by endothelial cells.³⁰ In addition, endothelial cells serve

FIG. 1. Hemostasis involves a balance of platelet activation. Too little platelet activation can lead to bleeding on disruption of vascular integrity. However, too much platelet activation can lead to thrombosis.

as a physical barrier between circulating platelets and the subendothelial matrix, including matrix components such as collagen, fibronectin, and von Willebrand Factor (vWF). On exposure to the subendothelial matrix, platelets adhere and become activated to form a hemostatic plug.³¹ After formation of this initial “platelet plug,” the coagulation cascade leads to fibrinogen-mediated platelet–platelet interactions, a hallmark of platelet aggregation. In addition, platelet spreading can occur to aid in closure of broken vasculature.

Platelets are activated by a variety of mediators, including secreted TXA₂ and prostaglandin E_2 , as well as by insults, such as reactive oxygen species (ROS) or exposure to damaged endothelial cells. An important hallmark of platelet activation involves shape change and granule secretion.¹² Importantly, release of granule contents leads to inflammation and immune cell recruitment in addition to further platelet activation. Platelet factor 4 (PF4), a granule component released on platelet activation, participates in atherogenesis. Another alpha granule component, CD62P (P-selectin), is a key cell surface molecule that aids in tethering activated platelets to monocytes, neutrophils, and lymphocytes.³² This interaction not only recruits immune cells to sites of vascular injury but also aids in neutrophil transmigration through the vessel wall and into the subendothelial space.³³

Moreover, platelets are the main source of soluble (s)CD40L³⁴ and abundantly release this mediator to recruit neutrophils and monocytes to the arterial intima and to activate the endothelium.³⁵ sCD40L is elevated in persons with CVD risk and in those who smoke.^{36,37} Platelets are also capable of synthesizing prostaglandins, including TXA₂ from arachidonic acid on stimulation, the release of which serves as a positive feedback loop in amplifying activation.

Another important aspect of platelet function is that they abundantly release different types of EVs into the circulation. Vesicle trafficking has changed our concept of gene regulation and protein signaling, as these small particles are capable of modifying recipient cells. EVs are found in blood, urine, saliva, breast milk, semen, and other body fluids and they are categorized according to their size.³⁸ Their cargo compositions and functions are determined by many factors, including but not limited to their cellular origin and an individual’s health status. Over the past decade,

EVs have been recognized as major players in inflammation and disease pathogenesis, shuttling functional proteins, lipids, and nucleic acids to neighboring³⁹ and distal cells to impact cancer and metastatic processes, CVD, and others.^{40–42}

Platelets release the majority of MPs resident in the circulation^{43,44} and release exosomes that are highly enriched with miRNA.²⁵ MPs are larger particles (size range 200–900 nm) that contain a host of procoagulant mediators, such as tissue factor and CD62P located on the surface of the membrane. Exosomes are smaller (size range 30–150 nm) and contain protein and RNA on their surface and within the lumen of the particle. Changes in MP and/or exosomal numbers or payload will provide important insights to predict inflammatory changes and subsequent cardiovascular risk in response to tobacco and non-tobacco product usage.

So what happens when the normal checks and balances that govern platelet function are lost? They become killers, eliciting unremitting inflammation and orchestrating thrombosis.

Platelets and cardiovascular disease

A key manifestation of CVD is atherosclerosis, a chronic inflammatory disease that can lead to MI or stroke. Atherosclerosis is characterized by the accumulation of cholesterol, lipids, and ultimately, plaques consisting of infiltrating neutrophils and macrophages in the cardiovascular system.⁴⁵ These plaques can rupture from the vessel wall, resulting in thrombus formation (e.g., a clot). Atherosclerosis is initiated by endothelial damage or dysfunction and is promoted by chronic inflammation and dyslipidemia, a condition of altered serum lipid levels.

In a proatherogenic serum lipid profile, triglycerides, low-density lipoprotein (LDL), and oxidized LDL (oxLDL) are increased, with a concomitant decrease in high-density lipoprotein (HDL).⁴⁶ When there is too much LDL or too little HDL, cholesterol and other lipids can be phagocytosed by macrophages, causing them to become lipid filled (termed foam cells). Foam cells lead to CVD by forming fatty streaks on vessel walls, recruiting monocytes through cytokine secretion and by creating fibrotic plaques within the vessel intima.⁴⁷ In addition, oxLDL directly activates the endothe-

lium and platelets, initiating the formation of a plaque and the beginning of CVD.

It is well known that platelets play a role in the acute stages of CVD associated with abnormal blood clotting (thrombosis) and vessel occlusion leading to an MI, stroke, and peripheral vascular disease.^{4,48,49} It is now realized that platelet dysregulation (e.g., hyper- or hypoactivity) plays a crucial role in the etiology and progression of CVD through potentiation of unwanted inflammatory and immunomodulatory mechanisms.^{4,45,48,50,51} Blood vessels can become injured by environmental causes, such as air pollution and smoking,^{52,53} or by other inflammatory pathologies. Repeated exposure to environmental stressors leads to aberrant signaling and responses by platelets.

Platelets are key mediators of hemostasis as well as inflammation, immunity, disease progression, wound healing, and resolution of inflammation.^{54–56} It is becoming increasingly apparent that platelets are much more complex than originally thought. Understanding how platelet activation is altered in response to exposure to environmental agents will help us to better understand how platelet dysregulation affects cardiovascular risk (Fig. 2).

Platelets are highly suited for *in vitro* and *ex vivo* studies

Hemostasis is a tightly regulated process and an imbalance between stimulatory and inhibitory signals can cause excessive or insufficient platelet activation, thus contributing to CVD and inflammation. In addition, platelets can be more or less sensitive to activation as a result of exposure to various chemical and environmental agents or in different disease states. Determining how platelets respond to chemical insults *in vivo* is complex. However, new techniques have allowed for rapid screening of platelets *in vitro* or *ex vivo*.

For example, blood platelets can be isolated from healthy human donors with a simple venipuncture and assessed for reactivity against various chemicals in 1 day (Fig. 3). Using a high-throughput 96-well or higher format, platelets can be treated with multiple doses of different chemicals, in various combinations, and with an array of activators. The platelets can then be assessed for expression of activation markers by flow cytometry, or the releasate can be

FIG. 2. Platelet activation. Platelets are smooth discoid-shaped cells in their inactive state and undergo shape change on activation. Platelets can be activated by vessel wall damage and other traditional hemostatic stimuli, foreign toxicants, such as cigarette smoke, as well as by inflammatory signals and ensuing CVD. Platelet microvesicles are shed from the surface of activated platelets, and various mediators are additionally secreted in response to stimulation. CD40L, CD40 ligand; CD62P, P-selectin; CVD, cardiovascular disease; PDGF, platelet-derived growth factor; PF4, platelet factor 4; RANTES, regulated on activation, normal T cell express and secreted; TGF- β , transforming growth factor beta.

FIG. 3. High-throughput *in vitro* platelet assays. Whole blood is collected from healthy human donors and centrifuged to obtain platelet-rich plasma. Either platelet-rich plasma or washed platelets are plated into a 96-well plate and treated with combinations of chemicals and activators. Cell-free supernatants can be analyzed for mediator release by immunoassay, and platelets can be evaluated for cell surface activation markers by flow cytometry.

examined using immunoassays, both of which can be done in various well formats. In this way, a large number of conditions can be tested on platelets from a single human donor.

In addition, platelets from multiple different human donors should be used to account for donor-to-donor variability in response to stimuli. Thus, performing experiments on freshly isolated primary cells from different donors will result in a more accurate representation of how various chemicals will affect human platelets from different subjects *in vivo*. Furthermore, blood platelets can be isolated from donors who are exposed to various tobacco products and their activation and function can be assessed *ex vivo* in the same high-throughput manner.

Due to the difficulty in obtaining fresh platelets in some settings, *in vitro* approaches using cell lines will also be beneficial. However, the current cell lines available for *in vitro* platelet work are not ideal. In the bone marrow, platelets are generated from megakaryocytes, where protrusions of the megakaryocyte are found extending into the circulation. Platelets are sheared off the ends of these protrusions by shear forces generated by flowing blood; thus, platelets are born.¹¹

In vitro, these shear forces are not present, and although platelet-like particles are generated, the composition and function of these “platelets” are not the same as *in vivo*. For example, *in vitro* generated platelets respond to stimuli, but do not spread on fibrinogen and only express some of the activation markers of their endogenously generated counterparts. Thus, it would be beneficial to create new megakaryocyte cell lines that produce platelets that better recapitulate bone marrow-derived platelets, as the use of *in vitro* platelets

from a single controlled cell line would increase reproducibility of data between different laboratories and even within the same laboratory across different days.

Platelets, Nicotine, and Tobacco

Effect of tobacco smoke on platelet function

Smoking and second-hand smoke enter the body via the mouth, nasal cavity, and lung. Exposure to platelets occurs by absorption into the circulation of components and volatiles, where components can have major influences on platelet function (Fig. 4). Cigarette smoking is a major risk factor for CVD, in part, because it activates platelets, endothelial cells, and monocytes.^{1,2,57,58} In fact, smokers have higher levels of circulating tissue factor and platelet-released proinflammatory molecules (e.g., sCD40L and platelet microvesicles), which are tied to dyslipidemia and platelet activation.^{2,26,49,59–61}

While there are more than 5,000 compounds in cigarette smoke, making it difficult to identify which molecules are associated with increased CVD. Some implicated molecules are ROS, which cause endothelial damage, LDL oxidation, and platelet activation. Some compounds that have been identified in smoke have profound effects on human cells, tissues, and systems. These include, but are not limited to known carcinogens, mono and aromatic amines, aldehydes, polycyclic aromatic hydrocarbons, carbon monoxide, and free radicals.

Smoke also contains electrophilic compounds that can induce platelet production through signaling of thrombopoiesis in the platelet progenitor cell, the megakaryocyte,⁶² and influence production and subsequent platelet function.⁶² For

FIG. 4. Platelet dysregulation by tobacco products. Tobacco products can alter many aspects of platelet function, including hemostasis, inflammation, and immunity, and disease pathology.

example, acrolein is a major constituent of smoke and a toxic factor that strongly binds to nucleophiles, such as glutathione. Consistent with the properties of other electrophiles, acrolein can form adducts with amino acid residues (e.g., cysteine) and was shown to increase protein adducts in platelets in mice.⁶³ This induced an increase in platelet aggregation, formation of platelet–leukocyte aggregates, and release of prothrombotic mediators from platelet granules (e.g., PF4). Acrolein toxicity is associated with increased CVD risk^{64,65} and predisposes to thrombotic risk.²

In addition to inflammatory and thrombotic markers described for platelets and CVD above, oxidative stress is a key measure of toxic response in platelets. Monoamine oxidase (MAO) A and B are mitochondrial enzymes that catalyze the oxidative deamination of amines and neurotransmitters.⁶⁶ Recent evidence suggests that MAO inhibitors are components of tobacco and tobacco products and contribute to smoking dependence.^{67,68} Interestingly, platelet MAOB activity is reduced in cigarette smokers and recovers after several weeks of smoking abstinence.^{67,69} Thus, MAOB may be a practical biomarker choice for evaluating oxidative response in platelets when testing next-generation products.

Nicotine and nicotine metabolites on platelet function

Nicotine is a highly addictive compound that is present in the tobacco leaf and other edible plants.⁷⁰ Nicotine in tobacco smoke is absorbed by blood vessels and quickly reaches the brain where it can act as both a stimulant and a relaxant, depending on the dose and duration of exposure.⁷¹ Nicotine is present in most tobacco products, such as cigarettes, cigars, pipe and chewing tobacco, snuff and snus. Once in the circulation, nicotine is extensively metabolized and eliminated within 2 hours. The major metabolite is cotinine (70%–80%), which is further processed to other compounds that include trans-3'-hydroxycotinine and others, which have longer half-lives and accumulate in the blood stream throughout the day.⁷²

Platelets have functional cholinergic signaling, expressing nicotinic acetylcholine receptors.^{73,74} However, there is significant disagreement with respect to the role of nicotine and nicotine metabolites on platelet function. Ljungberg et al. performed nicotine infusion studies to assess nicotine metabolism on platelet activity.⁷⁵ Their findings support *in vitro* studies performed by our group (unpublished data), demonstrating that nicotine and some metabolites do not overtly activate platelets. However, they found that nicotine exposure marginally inhibited platelet attachment to extracellular matrix (ECM) components, such as collagen. In contrast, our group has data demonstrating that nicotine exposure on human washed platelets did not affect platelet spreading on fibrinogen, but platelets exposed to cigarette smoke extract attenuated platelet spreading (Fig. 5).

The doses of nicotine used for our experiments were in the nanomolar range compared to micromolar used by Ljungberg et al., reaffirming the fact that nicotine and metabolites have differential actions and sensitivities that are dependent on the exposure environment. It should be noted that other studies have supported a role for platelet desensitization by nicotine^{76,77} and inhibition of platelet activity.⁷⁸ Long-term health effects from nicotine exposure have been difficult to determine, and nicotine studies with respect to nicotine replacement therapy are controversial.^{79,80}

Platelets and next-generation product assessment

With the advent of harm reduction and new guidelines giving the FDA the authority to regulate the marketing and composition of tobacco and alternative products, there is a significant need to establish rigorous scientific-based assessments of products. While there is a wealth of information regarding combustible tobacco products, investigations of smokeless tobacco, non-tobacco products, and next-generation products are lacking. An overview of tobacco and some alternative products, as well as the extent of FDA regulation of these products is provided in Table 1. Studies to assess alternative products, such as smokeless tobacco and electronic

FIG. 5. Tobacco products alter human platelet spreading. Washed platelets freshly isolated from healthy human donors were treated with vehicle (media alone), 100 nM nicotine, or 6.5 U/mL cigarette smoke extract for 30 minutes, then were allowed to spread on fibrinogen-coated coverslips for 45 minutes. Coverslips were washed with PBS, then fixed with 4% paraformaldehyde, and visualized with DIC microscopy. Cigarette smoke extracts were generated in the University of Rochester Smoke Inhalation Facility using 3R4F reference cigarettes (University of Kentucky) and an automated cigarette smoking machine (Jaeger-Baumgartner). DIC, differential interference contrast.

TABLE 1. TOBACCO AND ALTERNATIVE TOBACCO AND NON-TOBACCO PRODUCTS, AND CURRENT FDA REGULATIONS

<i>Product type</i>	<i>FDA regulation^a</i>
Smoke	
Cigarettes, commercial and roll your own	2009 ^b : Manufacture, import, packaging, labeling, advertising, promotion, sale, and distribution, including components, parts, and accessories
Cigars, filtered cigars, cigarillos	2016: Manufacture, import, packaging, labeling, advertising, promotion, sale, and distribution of cigars. This includes components and parts such as rolling papers and filters, but excludes accessories such as lighters and cutters
Pipe tobacco	2016: Manufacture, import, packaging, labeling, advertising, promotion, sale, and distribution of pipe tobacco. This includes components and parts such as pipes, but excludes accessories such as lighters
Hookah (waterpipes, shisha)	2016: Manufacture, import, packaging, labeling, advertising, promotion, sale, and distribution of hookah tobacco. This includes components and parts of newly regulated tobacco products but excludes accessories such as lighters, tongs, or external burners
Dissolvable tobacco products (some are considered smokeless products)	2016: Manufacture, import, packaging, labeling, advertising, promotion, sale, and distribution of all dissolvables. This includes components and parts but excludes accessories for newly regulated dissolvables that are not considered smokeless tobacco products
Smokeless	
Chewing tobacco, dry snuff, moist snuff or snus	2009 ^b : Manufacture, import, packaging, labeling, advertising, promotion, sale, and distribution of smokeless tobacco products, which includes products for use in the oral and nasal cavity
Non-tobacco	
ENDS (vaporizers, vape pens, hookah pens, electronic cigarettes)	2016: Manufacture, import, packaging, labeling, advertising, promotion, sale, and distribution of ENDS. This includes components and parts of ENDS, but excludes accessories
Nicotine gels (product that is absorbed via the skin)	2016: Manufacture, import, packaging, labeling, advertising, promotion, sale, and distribution of nicotine gels. This includes components and parts of newly regulated tobacco products but excludes accessories
Other modified risk products	
Heat not burn products	Not sold in the United States

^aFrom the FDA regulatory website, www.fda.gov

^bTobacco regulation by the FDA began in 2009 with the passage of the Family Smoking Prevention and Tobacco Control Act. ENDS, electronic nicotine delivery systems; FDA, US Food and Drug Administration.

nicotine delivery systems (ENDS), are lacking, underscoring the need to develop product evaluation methods to assess platelet function and in the context of CVD.

A recent *in vitro* study of platelet response to electronic cigarettes suggests that electronic vapor (E-vapor) negatively affects platelet function due to the fine particulate matter.⁷⁸ These studies examined platelet function tests that are dysregulated by traditional tobacco smoking, such as platelet aggregation and adhesion, and found that E-vapor also increased platelet activation, similarly to tobacco smoking.

A dearth of information regarding electronic cigarettes and platelets exists, underscoring the need for further research to study the effects of electronic cigarettes on platelet function. Testing will require the assessment of many different types of electronic cigarettes, as each has variable electrical power, which allows the user to customize the component mixture in the generated E-vapor. Equally challenging and vitally important will be testing the compositions of the many different electronic liquids from which E-vapor is generated (e.g., particulate composition, flavorings, and nicotine levels). These new assessments will benefit from both traditional platelet function tests and by new *in vitro* and *ex vivo* approaches, as described below.

***In Vitro* and *Ex Vivo* Approaches for Assessing Product Risk in Platelets**

Concurrent with new FDA guidelines, there has been a move to evaluate tobacco and alternative products, using *in vitro* and *ex vivo* biological models that may better reflect the human response to product exposure. While animal models offer the advantage of *in vivo* organismal studies and have proven valuable when human experimentation is unfeasible, the outcomes do not always extrapolate to human biology. It is important to note that animal studies have benefited both humans and animals, and mandatory considerations in experimental design have been implemented to minimize pain and suffering. However, there are still ethical considerations re-

garding the use of animals for scientific studies. Thus, further measures are merited to limit suffering and develop the best possible methods to reduce or eliminate animal testing in the future. Complex *in vitro* and *ex vivo* biological systems can and are being developed to investigate specific toxicological questions and product safety.

Surprisingly, platelets are a highly overlooked cell in these assessments, especially when considering their central dynamic role in inflammation, thrombosis, and all stages of CVD development. Moreover, human platelet studies are highly adaptable to human-based assessments. Platelets are numerous in the circulation and easy to isolate, and as such, represent a novel and translational method to assess inflammation and CVD risk in response to the next generation of tobacco and non-tobacco products.

A step by step approach to using platelets for assessment studies would encompass determining effects at the cell level and integrating these studies with more complex systems that account for cell to cell interactions, and finally to tissue/organ-level evaluation to bring relevancy to the actual health effects of a particular product. Taking this step by step approach using a variety of human-based systems allows for assessment of both acute and chronic effects, as well as the reversibility of a particular product once withdrawn. The methods outlined in Table 2 and described in detail below are standard methods that can be used with *in vitro* and *ex vivo* studies to measure and predict product toxicity and safety. These testing methods can easily be standardized and many are already used clinically in patient care.

Biomarker assessment and screening

There are many known biomarkers of inflammation, cardiovascular sequelae, and thrombosis, and many are also known to be dysregulated in response to tobacco product usage. Biomarkers in the context of platelet studies will be used to predict incidence or outcomes associated with tobacco and alternative tobacco product use. Biomarker selection

TABLE 2. EXAMPLES OF STANDARD TESTS FOR ASSESSMENT OF TOBACCO AND NON-TOBACCO PRODUCTS THAT ARE ADAPTABLE TO BOTH *IN VITRO* AND *EX VIVO* STUDIES

<i>Test</i>	<i>Method</i>	<i>Outcome measure</i>
Inflammatory biomarker screening		
Monoamine oxidase	Immunoassay (levels and activity)	Oxidative stress
sCD40L	Immunoassay	Platelet activation/thrombus stability
Custom-designed Luminex panels	Luminex™; immunoassay	Platelet and CVD biomarkers of inflammation and oxidative stress
P-selectin (CD62P)	Flow cytometry	Platelet activation, granule release
Platelet/leukocyte aggregates	Flow cytometry	Vascular inflammation
Extracellular vesicles	Flow cytometry/particle analyzers	Number, type, and content
Functional assays		
Aggregation	Luminescence, impedance	Coagulability, CVD
Platelet spreading	Extracellular matrices applied to surface	Surrogate measure of wound healing
Thromboelastography	Specialized instrumentation	Clot stability, clot lysis
Biological systems testing		
Dynamic flow chambers/microfluidics	Extracellular matrices applied to surface under flow conditions	Adhesion and thrombus stability under shear stress
Organs-on-a-chip	Three-dimensional multifunctional systems	Physiological response in organ system

CVD, cardiovascular disease.

will embody different signaling pathways that enlighten our understanding of which inflammatory or thrombotic pathways may be associated with changes induced or suppressed by a particular product and how multiple pathways may work in concert to promote disease pathology.

Table 2 provides a short list of potentially useful markers. For example, plasma levels of sCD40L are elevated in smokers.³⁷ Thus, it is expected that platelets release sCD40L in response to cigarette smoke extracts *in vitro*, as they are the main source of this CVD biomarker. Other key granule contents are released in response to tobacco smoke that include PF4 and thrombospondin, both associated with a procoagulant profile.⁸¹ Furthermore, expression of cell surface markers associated with platelet activation, such as CD62P, can be measured.

Platelet leukocyte aggregates (PLAs) are an important biomarker of vascular inflammation and are causally associated with the development and acceleration of atherosclerosis.⁸² Both the surface marker expression and PLAs can be measured using platelets that have been isolated and exposed to a particular product, extract, condensate, or compound or equally, platelets from product users can be assessed.

MAOB levels or activity may also be an important measure of product exposure, not only for combustible tobacco products but could also be an indicator of the effects of smokeless tobacco products on platelets.⁶⁷

Activation of cells can lead to higher levels of EV release and changes in vesicle content. EVs are stable in the circulation and thus can target cell types that are either proximal or quite distal to their origin of release. EVs can deliver specific cargo that is targeted to a particular cell type through ligand/receptor binding.⁸³ Pathologic changes in EV cargo and numbers can have profound effects on cell to cell communication, as well as autocrine signaling.

It is important for the assessment of next-generation products that increases in procoagulant EVs in response to tobacco smoke are documented.⁸⁴ Our group also has preliminary *ex vivo* evidence that vesicle release from platelets is increased in response to tobacco smoke (unpublished data). We expect that EV enumeration and characterization of platelet EV cargo represent a novel measure of changes in intercellular communication and will be predictive of whether a particular product is more or less harmful than traditional tobacco products.

Platelet function testing

Several platelet function tests are routinely used in the clinic and represent other experimental metrics that can be used to evaluate the influence of new products on platelet and vascular function. A basic test of platelet function used routinely is platelet aggregation. This test is most commonly performed on platelet-rich plasma (PRP) obtained from anticoagulated blood and measures the ability of platelets to change shape and form aggregates. This test can be used to assess hyperaggregability in response to product exposure and can also be performed with or without an inducing platelet agonist to characterize products that may be inhibitory to platelet function. In the case whereby platelet aggregation is increased by product exposure *in vivo*, the end cause could be vessel occlusion leading to MI, stroke, and heart damage.

Conversely, products that inhibit platelet activity may predispose individuals to bleeding. Thromboelastography is a

measure of coagulation efficiency that provides information regarding clot formation, strength, and stability, and can be used with PRP from product-free individuals and product users to determine effects to the platelet, as well as associated coagulation parameters.

Platelet spreading is an essential activity in wound healing and thrombosis. Following vascular injury, the exposed ECM signals platelets to form a plug. This requires the platelet to adhere to the site of trauma, flatten, and increase the area of contact (spread). Using platelet spreading as a surrogate model of injury repair, our group has shown that exposure to cigarette smoke extract causes a significant decrease in the platelet's ability to spread (Fig. 5). Platelet spreading is a quantifiable measure that provides information regarding the dynamics of adherence and spreading area. This technique can be performed in real time with isolated platelets to investigate the acute effects of exposure. Platelets can also be fixed and imaged at various time intervals following product exposure for investigation and quantification of chronic exposures.

Our group has found platelet spreading to be a valuable tool for investigating the effects of current tobacco products and it will provide a unique measure of how next-generation products may influence platelet function. New products may increase, decrease, or ideally, induce no change in this platelet measure. Inhibition or potentiation of platelet function would be indicative of platelet dysfunction. Standard protocols and harm risk assessment will need to be established.

Biological systems

Another consideration for studying exposure and toxicity assessment is the Cone and Plate(let) Analyzer, which was developed as a simple method to test platelet adhesion and aggregation under conditions that, while not perfect, better represent the *in vivo* environment.^{85–87} This method has shown utility in a variety of settings, including testing and monitoring of antiplatelet therapies and diagnostics.

Another type of testing that is valuable for *in vitro* product assessment allows for investigation of platelet function in a setting that more closely mimics the circulation. Microfluidics and dynamic flow chamber studies recapitulate blood vessel conditions, allowing for investigation of platelet interactions with other vascular cells and their binding of extracellular matrices under shear flow.^{88,89} These tests provide important quantifiable information regarding key biophysical properties of platelets and could be used to dissect the effects of exposure to tobacco or other next-generation products and whether they disrupt normal hemostatic processes.⁹⁰ Our group has previously performed these types of studies to evaluate the role of platelet NF κ B signaling in thrombus formation and stability.¹⁶

While it is difficult to mimic biological systems and organs *in vitro*, many new technical advances have greatly accelerated the development of improved and valid models. Dynamic flow and microfluidic chamber technologies are the basis for organs-on-a-chip, which are recently developed technologies that provide a new approach to study vascular disease and drug discovery.⁹ For example, a vascular organ-on-a-chip platform would be ideally suited to study thrombus formation, as tobacco products can increase the risk of embolism. Assessment of next-generation products using either whole blood or PRP would be possible at (1)

different shear rates, (2) with the application of different extracellular matrices (e.g., collagen, vWF, fibrinogen), and (3) would provide new insights into platelet–platelet interactions, as well as with erythrocytes, white blood cells, and endothelial cells. These types of assessments will be important in demonstrating whether or not next-generation products have reduced toxicity and disease risk profiles.

Considerations and challenges for *in vitro* testing

An important aspect of *in vitro* toxicological assessment will require optimal design of test methods and validation studies by integrating data from multiple biologic systems. Herein, we described several methods that could be used for the assessment of current and next-generation tobacco and alternative products using platelets in the context of CVD. The utilization of *in vitro* and *ex vivo* studies with human blood platelets circumvents many of the difficulties with data interpretation that are encountered using animal models and subsequent extrapolation of findings from animal to human systems.

Many of the proposed methods can be easily standardized and are adaptable to high-throughput technologies, which are important considerations for cost-effective and rigorous testing of products. As with any new approaches, there will be challenges, such as (1) developing hazard and risk thresholds to provide an accurate prediction of a product's risk to human health, (2) determining how diverse technologies and experimental endpoints can be combined to achieve an accepted regulatory testing regimen. One answer may lie in the integration of *in vitro* and *ex vivo* endpoints with bioinformatics and computational methods to broaden our understanding of the toxicological responses associated with specific biologic pathways, and (3) developing rigorous methods to compare different product types to inform the public of the potential risks or risk reduction.⁷

Conclusions

There is a definite need for new approaches to evaluate existing and next-generation tobacco and non-tobacco products that will provide information about health risks and the mechanistic underpinnings contributing to disease pathology. Platelets are major hemostatic regulators and important instigators of vascular inflammation and dysregulation inherent in CVD development and progression, and thrombosis. Platelet-based *in vitro* tests/assays of the effects of tobacco, nicotine, and next-generation products can be robustly designed that are a simple, comprehensive, and integrated means to assess platelet function and cardiovascular health status. There are fundamental problems and limitations associated with any new research approach. However, the advantage using *in vitro* translational human biological systems is that the resulting findings will better reflect the human condition, which will lead to better predictions of human risk and novel approaches to reduce harm.

Acknowledgments

This work was supported, in part, by T90 DE21985, P30 ES001247, R21 HL128129, T32 ES007026 from the National Institutes of Health, UL1TR000042 from the National Center for Research Resources (NCRR), and by the University of Rochester Center for AIDS Research

Grant P30AI078498 (NIH/NIAID) and the University of Rochester School of Medicine and Dentistry, and GM068411 from the Institutional Ruth L. Kirschstein National Research Service Award. The contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIH or NCRR.

Author Disclosure Statement

No competing financial interests exist.

References

1. Messner B, Bernhard D. Smoking and cardiovascular disease: Mechanisms of endothelial dysfunction and early atherogenesis. *Arterioscler Thromb Vasc Biol* 2014;34:509–515.
2. Csordas A, Bernhard D. The biology behind the atherothrombotic effects of cigarette smoke. *Nat Rev Cardiol* 2013;10:219–230.
3. Weyrich AS, Lindemann S, Zimmerman GA. The evolving role of platelets in inflammation. *J Thromb Haemost* 2003;1:1897–1905.
4. Huo Y, Ley KF. Role of platelets in the development of atherosclerosis. *Trends Cardiovasc Med* 2004;14:18–22.
5. Weyrich AS, Zimmerman GA. Platelets: Signaling cells in the immune continuum. *Trends Immunol* 2004;25:489–495.
6. Family Smoking Prevention and Tobacco Control Act, Pre-amble, Public L. No. 111-131, 2009.
7. Azzopardi D, Patel K, Jaunky T, et al. Electronic cigarette aerosol induces significantly less cytotoxicity than tobacco smoke. *Toxicol Mech Methods* 2016;26:477–491.
8. Hartung T, Daston G. Are *in vitro* tests suitable for regulatory use? *Toxicol Sci* 2009;111:233–237.
9. Bhise NS, Ribas J, Manoharan V, et al. Organ-on-a-chip platforms for studying drug delivery systems. *J Control Release* 2014;190:82–93.
10. Rondina MT, Weyrich AS, Zimmerman GA. Platelets as cellular effectors of inflammation in vascular diseases. *Circ Res* 2013;112:1506–1519.
11. Avraham H. Regulation of megakaryocytopoiesis. *Stem Cells* 1993;11:499–510.
12. Koseoglu S, Flaumenhaft R. Advances in platelet granule biology. *Curr Opin Hematol* 2013;20:464–471.
13. Rowley JW, Oler AJ, Tolley ND, et al. Genome-wide RNA-seq analysis of human and mouse platelet transcriptomes. *Blood* 2011;118:e101–e111.
14. Weyrich AS, Dixon DA, Pabla R, et al. Signal-dependent translation of a regulatory protein, Bcl-3, in activated human platelets. *Proc Natl Acad Sci U S A* 1998;95:5556–5561.
15. Lindemann S, Tolley ND, Dixon DA, et al. Activated platelets mediate inflammatory signaling by regulated interleukin 1 β synthesis. *J Cell Biol* 2001;154:485–490.
16. Spinelli SL, Casey AE, Pollock SJ, et al. Platelets and megakaryocytes contain functional nuclear factor- κ B. *Arterioscler Thromb Vasc Biol* 2010;30: 591–598.
17. Spinelli SL, Maggirwar SB, Blumberg N, et al. Nuclear emancipation: A platelet tour de force. *Sci Signal* 2010;3:pe37.
18. Liu F, Morris S, Epps J, et al. Demonstration of an activation regulated NF- κ B/I- κ B α complex in human platelets. *Thromb Res* 2002;106:199–203.
19. Malaver E, Romaniuk MA, D'Atri LP, et al. NF- κ B inhibitors impair platelet activation responses. *J Thromb Haemost* 2009;7:1333–1343.
20. Gambaryan S, Kobsar A, Rukoyatkin N, et al. Thrombin and collagen induce a feedback inhibitory signaling pathway in platelets involving dissociation of the catalytic

- subunit of protein kinase A from an NF κ B-I κ B complex. *J Biol Chem* 2010;285:18352–18363.
21. Akbiyik F, Ray DM, Gettings KF, et al. Human bone marrow megakaryocytes and platelets express PPAR γ , and PPAR γ agonists blunt platelet release of CD40 ligand and thromboxanes. *Blood* 2004;104:1361–1368.
  22. Ray DM, Spinelli SL, Pollock SJ, et al. Peroxisome proliferator-activated receptor gamma and retinoid X receptor transcription factors are released from activated human platelets and shed in microparticles. *Thromb Haemost* 2008;99:86–95.
  23. Lannan KL, Sahler J, Kim N, et al. Breaking the mold: Transcription factors in the anucleate platelet and platelet-derived microparticles. *Front Immunol* 2015;6:48.
  24. Yoon YJ, Kim OY, Gho YS. Extracellular vesicles as emerging intercellular communicasomes. *BMB Rep* 2014;47:531–539.
  25. Landry P, Plante I, Ouellet DL, et al. Existence of a micro-RNA pathway in anucleate platelets. *Nat Struct Mol Biol* 2009;16:961–966.
  26. Morrell CN, Aggrey AA, Chapman LM, et al. Emerging roles for platelets as immune and inflammatory cells. *Blood* 2014;123:2759–2767.
  27. Garraud O, Hamzeh-Cognasse H, Pozzetto B, et al. Bench-to-bedside review: Platelets and active immune functions—New clues for immunopathology? *Crit Care* 2013;17:236.
  28. Ruggeri ZM. Platelets in atherothrombosis. *Nat Med* 2002;8:1227–1234.
  29. Thomas MR, Storey RF. The role of platelets in inflammation. *Thromb Haemost* 2015;114:449–458.
  30. Radomski MW, Palmer RM, Moncada S. Comparative pharmacology of endothelium-derived relaxing factor, nitric oxide and prostacyclin in platelets. *Br J Pharmacol* 1987;92:181–187.
  31. Hawiger J. Formation and regulation of platelet and fibrin hemostatic plug. *Hum Pathol* 1987;18:111–122.
  32. Yeo EL, Sheppard JA, Feuerstein IA. Role of P-selectin and leukocyte activation in polymorphonuclear cell adhesion to surface adherent activated platelets under physiologic shear conditions (an injury vessel wall model). *Blood* 1994;83:2498–2507.
  33. Lam FW, Burns AR, Smith CW, et al. Platelets enhance neutrophil transendothelial migration via P-selectin glycoprotein ligand-1. *Am J Physiol Heart Circ Physiol* 2011;300:H468–H475.
  34. André P, Nannizzi-Alaimo L, Prasad SK, et al. Platelet-derived CD40L: The switch-hitting player of cardiovascular disease. *Circulation* 2002;106:896–899.
  35. Phipps RP. Atherosclerosis: The emerging role of inflammation and the CD40-CD40 ligand system. *Proc Natl Acad Sci U S A* 2000;97:6930–6932.
  36. Kaufman J, Spinelli SL, Schultz E, et al. Release of biologically active CD154 during collection and storage of platelet concentrates prepared for transfusion. *J Thromb Haemost* 2007;5:788–796.
  37. Garlachs CD, Cicha I, Raaz D, et al. CD40/CD154 system and pro-inflammatory cytokines in young healthy male smokers without additional risk factors for atherosclerosis. *Inflamm Res* 2009;58:306–311.
  38. Lötvall J, Hill AF, Hochberg F, et al. Minimal experimental requirements for definition of extracellular vesicles and their functions: A position statement from the International Society for Extracellular Vesicles. *J Extracell Vesicles* 2014;3:26913.
  39. Lo Cicero A, Stahl PD, Raposo G. Extracellular vesicles shuffling intercellular messages: For good or for bad. *Curr Opin Cell Biol* 2015;35:69–77.
  40. De Toro J, Herschlik L, Waldner C, et al. Emerging roles of exosomes in normal and pathological conditions: New insights for diagnosis and therapeutic applications. *Front Immunol* 2015;6:203.
  41. Inal JM, Fairbrother U, Heugh S. Microvesiculation and disease. *Biochem Soc Trans* 2013;41:237–240.
  42. Yuana Y, Sturk A, Nieuwland R. Extracellular vesicles in physiological and pathological conditions. *Blood Rev* 2013;27:31–39.
  43. Burnier L, Fontana P, Kwak BR, et al. Cell-derived microparticles in haemostasis and vascular medicine. *Thromb Haemost* 2009;101:439–451.
  44. Horstman LL, Ahn YS. Platelet microparticles: a wide-angle perspective. *Crit Rev Oncol Hematol* 1999;30:111–142.
  45. Lindemann S, Krämer B, Seizer P, et al. Platelets, inflammation and atherosclerosis. *J Thromb Haemost* 2007;5(Suppl 1):203–211.
  46. Merched A, Ko K, Gotlinger K, et al. Atherosclerosis: Evidence for impairment of resolution of vascular inflammation governed by specific lipid mediators. *FASEB J* 2008;22:3595–3606.
  47. Friedman M, Byers SO, St George S. Site of origin of the luminal foam cells of atherosclerosis. *Am J Clin Pathol* 1966;45:238–246.
  48. Dong ZM, Chapman SM, Brown AA, et al. The combined role of P- and E-selectins in atherosclerosis. *J Clin Invest* 1998;102:145–152.
  49. Shi G, Morrell CN. Platelets as initiators and mediators of inflammation at the vessel wall. *Thromb Res* 2011;127:387–390.
  50. Spinelli SL, O'Brien JJ, Bancos S, et al. The PPAR-platelet connection: Modulators of inflammation and potential cardiovascular effects. *PPAR Res* 2008;2008:328172.
  51. Daub K, Langer H, Seizer P, et al. Platelets induce differentiation of human CD34+ progenitor cells into foam cells and endothelial cells. *FASEB J* 2006;20:2559–2561.
  52. Rückerl R, Hampel R, Breitner S, et al. Associations between ambient air pollution and blood markers of inflammation and coagulation/fibrinolysis in susceptible populations. *Environ Int* 2014;70:32–49.
  53. Edirisinghe I, Yang SR, Yao H, et al. VEGFR-2 inhibition augments cigarette smoke-induced oxidative stress and inflammatory responses leading to endothelial dysfunction. *FASEB J* 2008;22:2297–2310.
  54. Henn V, Slupsky JR, Gräfe M, et al. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature* 1998;391:591–594.
  55. Sprague DL, Sowa JM, Elzey BD, et al. The role of platelet CD154 in the modulation in adaptive immunity. *Immunol Res* 2007;39:185–193.
  56. Semple JW, Freedman J. Platelets and innate immunity. *Cell Mol Life Sci* 2010;67:499–511.
  57. Barua RS, Sy F, Srikanth S, et al. Acute cigarette smoke exposure reduces clot lysis—Association between altered fibrin architecture and the response to t-PA. *Thromb Res* 2010;126:426–430.
  58. Critchley JA, Capewell S. Mortality risk reduction associated with smoking cessation in patients with coronary heart disease: A systematic review. *JAMA* 2003;290:86–97.
  59. Blumberg N, Heal JM, Gettings KF, et al. An association between decreased cardiopulmonary complications (transfusion-related acute lung injury and transfusion-

- associated circulatory overload) and implementation of universal leukoreduction of blood transfusions. *Transfusion* 2010;50:2738–2744.
60. McNicol A, Israels SJ. Beyond hemostasis: The role of platelets in inflammation, malignancy and infection. *Cardiovasc Hematol Disord Drug Targets* 2008;8:99–117.
  61. Neubauer H, Setiadi P, Pinto A, et al. Upregulation of platelet CD40, CD40 ligand (CD40L) and P-selectin expression in cigarette smokers: A flow cytometry study. *Blood Coagul Fibrinolysis* 2009;20:694–698.
  62. O'Brien JJ, Spinelli SL, Tober J, et al. 15-deoxy-(Delta)12,14-PGJ2 enhances platelet production from megakaryocytes. *Blood* 2008;112:4051–4060.
  63. Sithu SD, Srivastava S, Siddiqui MA, et al. Exposure to acrolein by inhalation causes platelet activation. *Toxicol Appl Pharmacol* 2010;248:100–110.
  64. DeJarnett N, Conklin DJ, Riggs DW, et al. Acrolein exposure is associated with increased cardiovascular disease risk. *J Am Heart Assoc* 2014;3. pii: e000934.
  65. Glynn MF, Mustard JF, Buchanan MR, et al. Cigarette smoking and platelet aggregation. *Can Med Assoc J* 1966;95:549–553.
  66. Edmondson DE, Mattevi A, Binda C, et al. Structure and mechanism of monoamine oxidase. *Curr Med Chem* 2004;11:1983–1993.
  67. Hogg RC. Contribution of monoamine oxidase inhibition to tobacco dependence: A review of the evidence. *Nicotine Tob Res* 2016;18:509–523.
  68. Berlin I, Anthenelli RM. Monoamine oxidases and tobacco smoking. *Int J Neuropsychopharmacol* 2001;4:33–42.
  69. Guillem K, Vouillac C, Azar MR, et al. Monoamine oxidase inhibition dramatically increases the motivation to self-administer nicotine in rats. *J Neurosci* 2005;25:8593–8600.
  70. Siegmund B, Leitner E, Pfannhauser W. Determination of the nicotine content of various edible nightshades (Solanaceae) and their products and estimation of the associated dietary nicotine intake. *J Agric Food Chem* 1999;47:3113–3120.
  71. Yildiz D. Nicotine, its metabolism and an overview of its biological effects. *Toxicol* 2004;43:619–632.
  72. Papaseit E, Farré M, Graziano S, et al. Monitoring nicotine intake from e-cigarettes: Measurement of parent drug and metabolites in oral fluid and plasma. *Clin Chem Lab Med* 2016 [Epub ahead of print]; DOI: 10.1515/cclm-2016-0405.
  73. Schedel A, Thornton S, Schloss P, et al. Human platelets express functional alpha7-nicotinic acetylcholine receptors. *Arterioscler Thromb Vasc Biol* 2011;31:928–934.
  74. Schedel A, Kaiser K, Uhlig S, et al. Megakaryocytes and platelets express nicotinic acetylcholine receptors but nicotine does not affect megakaryopoiesis or platelet function. *Platelets* 2016;27:43–50.
  75. Ljungberg LU, Persson K, Eriksson AC, et al. Effects of nicotine, its metabolites and tobacco extracts on human platelet function in vitro. *Toxicol In Vitro* 2013;27:932–938.
  76. Chahine R, Calderone A, Navarro-Delmas C. The in vitro effects of nicotine and cotinine on prostacyclin and thromboxane biosynthesis. *Prostaglandins Leukot Essent Fatty Acids* 1990;40:261–266.
  77. Rubenstein D, Jesty J, Bluestein D. Differences between mainstream and sidestream cigarette smoke extracts and nicotine in the activation of platelets under static and flow conditions. *Circulation* 2004;109:78–83.
  78. Hom S, Chen L, Wang T, et al. Platelet activation, adhesion, inflammation, and aggregation potential are altered in the presence of electronic cigarette extracts of variable nicotine concentrations. *Platelets* 2016;27:694–702.
  79. Benowitz NL, Gourlay SG. Cardiovascular toxicity of nicotine: Implications for nicotine replacement therapy. *J Am Coll Cardiol* 1997;29:1422–1431.
  80. Lee PN, Fariss MW. A systematic review of possible serious adverse health effects of nicotine replacement therapy. *Arch Toxicol* 2016 [Epub ahead of print]; DOI: 10.1007/s00204-016-1856-y.
  81. Caponnetto P, Russo C, Di Maria A, et al. Circulating endothelial-coagulative activation markers after smoking cessation: A 12-month observational study. *Eur J Clin Invest* 2011;41:616–626.
  82. Sarma J, Laan CA, Alam S, et al. Increased platelet binding to circulating monocytes in acute coronary syndromes. *Circulation* 2002;105:2166–2171.
  83. Maas SL, Breakefield XO, Weaver AM. Extracellular vesicles: Unique intercellular delivery vehicles. *Trends Cell Biol* 2016 [Epub ahead of print]; DOI: <http://dx.doi.org/10.1016/j.tcb.2016.11.003>.
  84. Li M, Yu D, Williams KJ, et al. Tobacco smoke induces the generation of procoagulant microvesicles from human monocytes/macrophages. *Arterioscler Thromb Vasc Biol* 2010;30:1818–1824.
  85. Savion N, Varon D. Impact—The cone and plate(let) analyzer: Testing platelet function and anti-platelet drug response. *Pathophysiol Haemost Thromb* 2006;35:83–88.
  86. Shenkman B, Savion N, Dardik R, et al. Testing of platelet deposition on polystyrene surface under flow conditions by the cone and plate(let) analyzer: Role of platelet activation, fibrinogen and von Willebrand factor. *Thromb Res* 2000;99:353–361.
  87. Varon D, Dardik R, Shenkman B, et al. A new method for quantitative analysis of whole blood platelet interaction with extracellular matrix under flow conditions. *Thromb Res* 1997;85:283–294.
  88. Mody NA, King MR. Platelet adhesive dynamics. Part I: Characterization of platelet hydrodynamic collisions and wall effects. *Biophys J* 2008;95:2539–2555.
  89. Strazza M, Azoulay-Alfaguter I, Peled M, et al. Assay of adhesion under shear stress for the study of T lymphocyte-adhesion molecule interactions. *J Vis Exp* 2016 [Epub ahead of print]; DOI: 10.3791/54203.
  90. Mody NA, King MR. Platelet adhesive dynamics. Part II: High shear-induced transient aggregation via GPIIb/IIIa-vWF-GPIIb/IIIa bridging. *Biophys J* 2008;95:2556–2574.

Address correspondence to:

Dr. Richard P. Phipps

Department of Environmental Medicine

University of Rochester School of Medicine and Dentistry

601 Elmwood Avenue

Box 850

Rochester, NY 14642

E-mail: richard_phipps@urmc.rochester.edu