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

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## 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]

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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 ($2–4 \times 10^8$/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 $A_2$ ($TXA_2$) 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$\kappa$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 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.

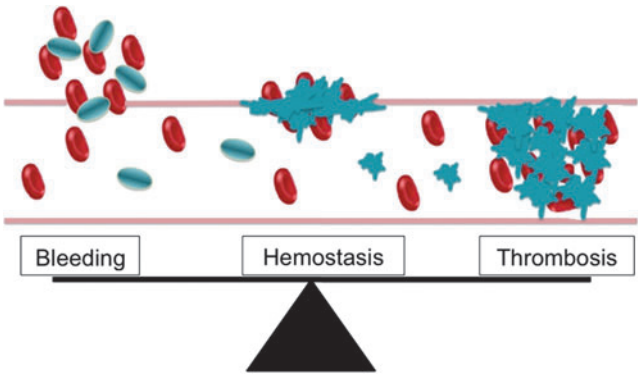


**FIG. 1.** Hemostasis involves a balance of platelet activation. Too little platelet activation can lead to bleeding on disruption of vascular integrity. However, too much platelet activation can lead to thrombosis.

Platelets are activated by a variety of mediators, including secreted $TXA_2$ 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)CD40L[34] 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 $TXA_2$ 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 neighboring[39] 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 cardiovasculature.[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 endothelium 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

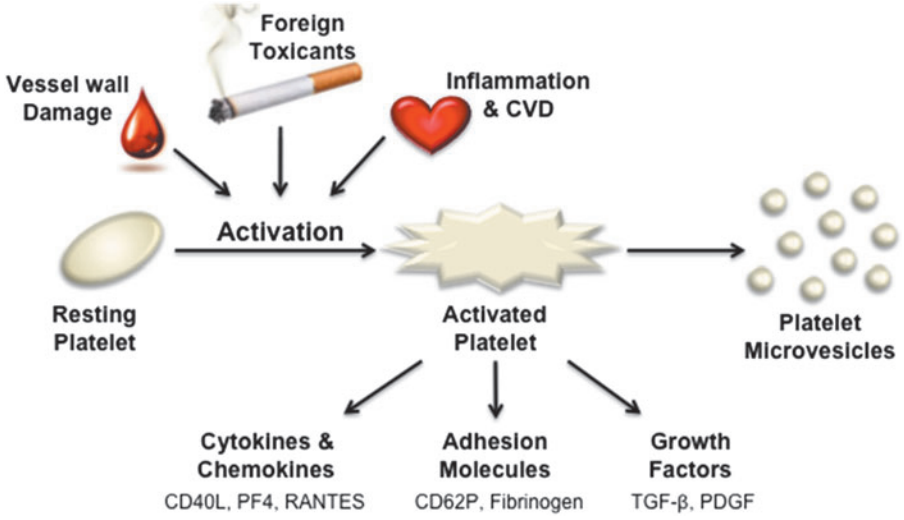


**FIG. 2.** Platelet activation. Platelets are smooth discoid-shaped cells in their inactive state and undergo shape change on activation. Platelets can be activated by vessel wall damage and other traditional hemostatic stimuli, foreign toxicants, such as cigarette smoke, as well as by inflammatory 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-$\beta$, transforming growth factor beta.

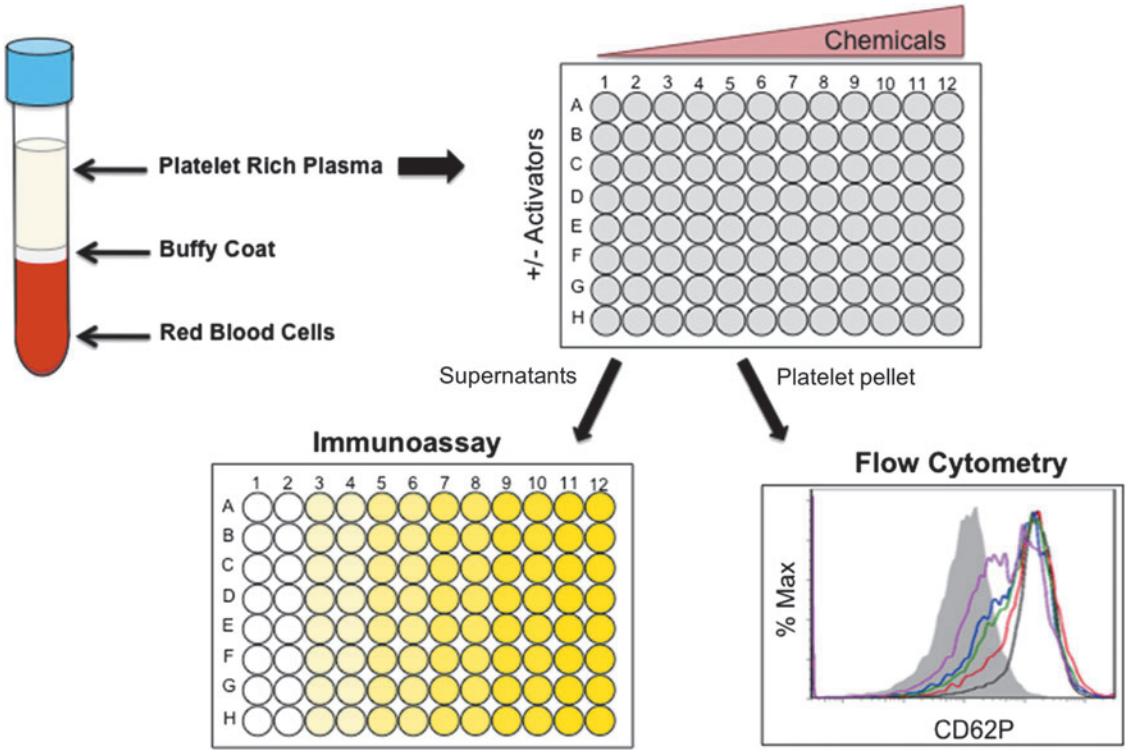


**FIG. 3.** High-throughput *in vitro* platelet assays. Whole blood is collected from healthy human donors and centrifuged to obtain platelet-rich plasma. Either platelet-rich plasma or washed platelets are plated into a 96-well plate and treated with combinations of chemicals and activators. Cell-free 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

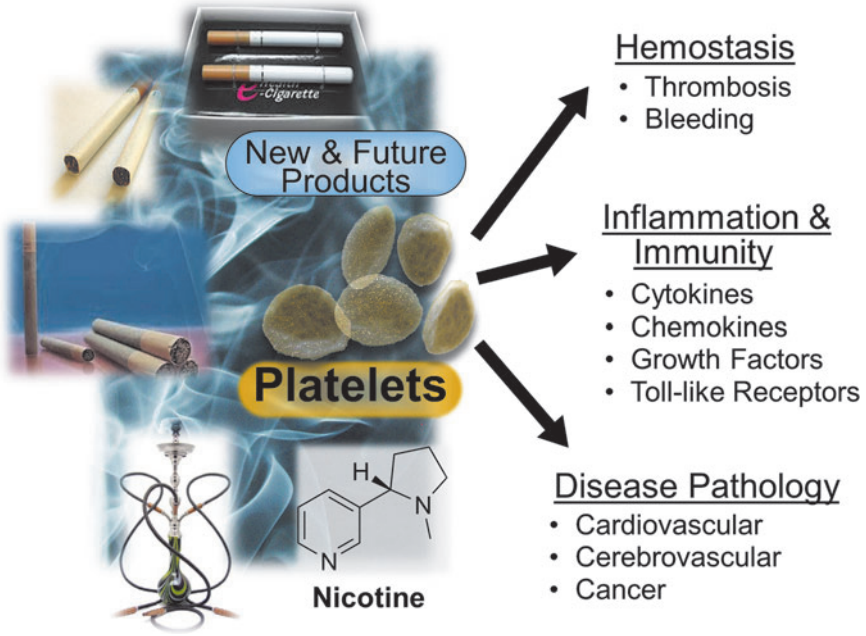


**FIG. 4.** Platelet dysregulation by tobacco products. Tobacco products can alter many aspects of platelet function, including hemostasis, inflammation, and immunity, and disease pathology.

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

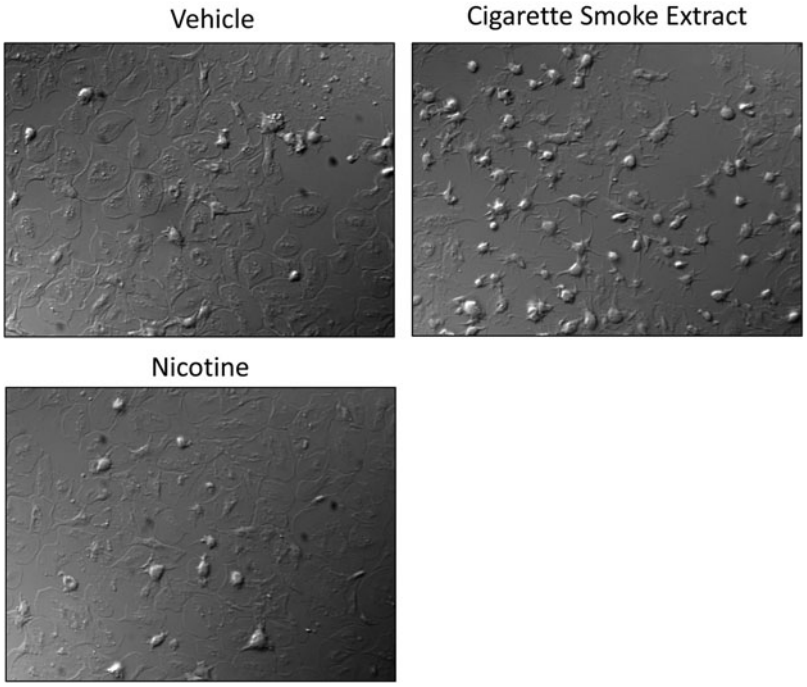


**FIG. 5.** Tobacco products alter human platelet spreading. Washed platelets freshly isolated from healthy human donors were treated with vehicle (media alone), 100 nM nicotine, or 6.5 U/mL cigarette smoke extract for 30 minutes, then were allowed to spread on fibrinogen-coated coverslips for 45 minutes. 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

| *Product type* | *FDA regulation*[a] |
|---|---|
| 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 |

[a]From the FDA regulatory website, www.fda.gov
[b]Tobacco 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 regarding 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

| *Test* | *Method* | *Outcome measure* |
|---|---|---|
| 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, impedience | 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 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$\kappa$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.

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## Author Disclosure Statement

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