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# The Effect Of Waterpipe/Hookah On Platelet Function And The Impact Of Inhibition Of Gβγ Subunits On Hemostasis And Thrombogenesis

Ahmed Alarabi
*University of Texas at El Paso*

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AHMED BILKHAYR ALARABI

Doctoral Program in Interdisciplinary Health Sciences

APPROVED:

Fadi T. Khasawneh, Ph.D. Chair

Fatima Z. Alshbool, PharmD, Ph.D. Co-Chair

Zubair A. Karim, Ph.D.

Mahesh Narayan, Ph.D.

Stephen L Crites, Jr., Ph.D.
Dean of the Graduate School

## Dedication

I dedicate my dissertation work to my family who provided me with unconditional support and love.

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by

AHMED BILKHAYR ALARABI, MD, MPH, DOMH

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# Abstract (Hookah)

Cardiovascular diseases (CVD's) is a major public health problem. Among CVD's risk factors, tobacco smoking is considered the single most preventable cause of death, with thrombosis being the main mechanism of CVD's mortality in smokers. While tobacco smoking has been on the decline, the use of waterpipe/hookah has been rising, mainly due to the perception that they are "less harmful" than regular cigarettes. Strikingly, there are few studies on the negative effects of waterpipes on the cardiovascular system, and none regarding their direct contribution to thrombus formation. We employed a waterpipe whole-body exposure protocol that mimics real-life human exposure scenarios, and investigated its effects, relative to clean air, on platelet function, hemostasis and thrombogenesis. We found that both adults as well as *in utero* waterpipe smoke (WPS) exposed mice exhibited shortened thrombus occlusion and bleeding times. Further, our results show that platelets from adults and *in utero* WPS exposed mice are hyperactive, with enhanced agonist-induced aggregation, dense and α-granule secretion, αIIbβ3 (GPIIb/IIIa) integrin activation, phosphatidylserine expression and platelet spreading, when compared with clean air-exposed platelets. Finally, at the molecular level, it was found that Akt and ERK phosphorylation are enhanced in the adult WPS exposed-, and in nicotine-treated platelets. Our findings demonstrate, for the first time, that adult and *in utero* WPS exposure directly modulate hemostasis and increases the risk of thrombosis, and that this is mediated, in part, via a state of platelet hyperactivity. The negative health impact of WPS/hookah, therefore, should not be underestimated. Moreover, this study should also help in raising public awareness of the toxic effects of waterpipe/hookah.

# Abstract (Gβγ subunits)

Platelet activation involves tightly regulated processes to ensure a proper hemostasis response, but when unbalanced, it can lead to pathological consequences such as thrombus formation. G-protein coupled receptors (GPCRs) regulate platelet function by mediating the response to various physiological agonists. To this end, an essential mediator of GPCR signaling is the G protein Gαβγ heterotrimers, and the βγ subunits are central players in downstream signaling pathways. While much is known regarding the role of the Gα subunit in platelet function, that of the βγ remains poorly understood. Therefore, we investigated the role of Gβγ subunits in platelet function using a Gβγ (small molecule) inhibitor, namely gallein. We observed that gallein inhibits platelet aggregation and secretion in response to agonist stimulation, in both mouse and human platelets. Furthermore, gallein also exerted inhibitory effects on integrin αIIbβ3 activation and clot retraction. Finally, gallein's inhibitory effects manifested *in vivo,* as documented by its ability to impair physiological hemostasis and delay thrombus formation. Taken together, our findings demonstrate, for the first time, that Gβγ directly regulates GPCR-dependent platelet function, *in vitro* and *in vivo*. Moreover, these data highlight Gβγ as a novel therapeutic target for managing thrombotic disorders.

Table of Contents

List of Tables

List of Figures

# Chapter I: Introduction

## Cardiovascular Diseases

According to the world health organization (WHO), cardiovascular diseases (CVDs) are the leading cause of death globally, taking an estimated 17.9 million lives each year. Generally, CVDs are a group of disorders of the heart and blood vessels and include coronary heart disease, cerebrovascular disease, rheumatic heart disease, and other heart conditions. In the united states (US) CVDs remain the leading cause of death with a death toll of 840,768 deaths (635,260 cardiac) in 2016 (Benjamin et al. 2019). In addition, CVDs are tied to a huge economic burden with a yearly total cost of $351.2 billion in 2014-2015, with $213.8 billion in direct cost, including 46% for inpatient care. These numbers make CVDs of huge public health concern and an issue that need continuous attention. Among CVDs, coronary heart disease (CHD) constitutes (43.2%) and is the leading cause of death from CVDs followed by stroke with (16.9%) (Benjamin et al. 2019). Both CHD and stroke are thrombotic based cardiovascular diseases in which the process of thrombosis plays a major role in their pathogenesis (Raskob et al. 2014). It is well established that platelets play a major role in the process of thrombosis, and antiplatelet drugs have long been shown to be beneficial for treatment and prevention of acute coronary events. Therefore, understanding platelets and their function have become very important. We will discuss platelets and their function in the next section.

## Platelets (Origin, Morphology, and Function)

Platelets are small (1-3μm) desk shaped cells that originate from the megakaryocyte cells in the bone marrow (Patel, Hartwig, and Italiano 2005). These small cells have a short life span of 7-10 days and the majority (2/3) of platelets are stored in the spleen. Platelets serve a very essential function which is hemostasis (the ability of the vascular system to maintain its own integrity and

stop blood loss). During normal condition, platelets circulate in a resting state, however, when triggered by vascular injury, platelets are rapidly activated and aggregate to form a primary hemostatic plug (platelet plug) on the vessel wall, which along with coagulation cascade help seal the vascular leakage, and as consequence prevent blood loss (Gremmel, Frelinger, and Michelson 2016). Hemostasis is a well regulated process in which any disruption to this process can lead to an unwanted extension of the platelet plug, which results in a pathological thrombosis (Packham 1994) (occlusion of blood flow to vital organs/tissues by the pathological thrombi). Occlusion of blood flow to an organ can lead to oxygen deprivation (hypoxia), in which if it's long enough can consequently lead to tissue death.

**Primary Hemostatic Plug (Platelet Plug)**

Normal endothelium of blood vessels helps keep platelets in resting state by releasing different substances that inhibit platelet activity, and that maintains blood in a fluid state (Yau, Teoh, and Verma 2015). When a break/damage happens to the endothelium, the subendothelial matrix will be exposed and therefore platelets start to gravitate toward the exposed matrix (e.g., collagen) and adhere to it in a process called adhesion. Consequently, platelets get activated and that leads to secretion of substances stored inside their granules to the outside local environment to help in recruiting other platelets to clump together in a process called aggregation, and this will lead to the formation of the primary hemostatic plug (platelet plug). At this stage, the primary hemostatic plug is unstable and therefore, the secondary hemostatic plug takes place through the coagulation cascade to transform the soluble fibrinogen to insoluble fibrin. This insoluble fibrin forms a network mesh around the platelet plug, which serves to strengthen and stabilize the blood clot. Both the primary hemostatic plug (platelet plug) and the coagulation cascade processes happen simultaneously and are mechanistically intertwined (Gale 2011).

**Platelet Signaling (Agonists and Receptors)**

On the cellular level, the ability of platelets to adhere to the exposed subendothelial matrix and to aggregate is mediated by a number of receptors, agonists, adhesive proteins and plasma-derived adhesive proteins. One of the most important receptors in the process of adhesion is GPIb-IX-V receptor which binds to von Willebrand factor (vWF), an important step for the adhesion with the subendothelial collagen matrix under the sheer flow. GPVI is another receptor that is activated by collagen and is important for platelet adhesion and subsequent activation (Gale 2011).

Platelet integrin activation is mandatory for the process of aggregation. In particular αIIbβ3, α2β1 and αvβ3, which are normally present on the platelet surface in an inactive form, but platelet activation induces conformational changes and that exposes ligand binding sites (Luo and Springer 2006, Xiao et al. 2004). Of all the integrins in platelets, αIIbβ3 is the most important of these receptors as it is present in abundance on the platelet surface. In addition, αIIbβ3 binds to multiple ligands that promote platelet-platelet aggregation including fibrinogen, VWF, collagen, fibronectin and vitronectin (Varga-Szabo, Pleines, and Nieswandt 2008).

Feedback activation of the locally available platelets surrounding the site of injury is critical for further aggregation and propagation of the platelet plug. This activation is mainly mediated by agonists released by activated platelets themselves acting on G protein-coupled receptors (GPCRs). Of those, adenosine diphosphate (ADP) is released from platelet dense granules and binds to its receptors, namely P2Y1 and P2Y12 (Mills 1996). Thromboxane $A_2$ is synthesized de novo by activated platelets and binds to the thromboxane receptor (TPR) on platelets (Hanasaki and Arita 1988). Furthermore, serotonin is also secreted from dense granules and contributes to platelet activation through 5HT2A receptors. The collective results of the organized work of this network of receptors and agonists are the formation of the primary

hemostatic plug. One important step in platelet activation that is linked to the formation of the secondary platelets plug is the formation of thrombin. Thrombin is the terminal serine protease product of the coagulation cascade. Thrombin cleaves 2 protease-activated receptors (PARs) on human platelets, PAR1 and PAR4. These are also G protein-coupled receptors (GPCRs), and cleavage by thrombin exposes a new N-terminus that serves as a tethered ligand to activate the receptor (Kahn et al. 1998, Vu et al. 1991). All of these receptors transduce signaling which ultimately results in platelet granule secretion, integrin activation and platelet cytoskeleton changes that are needed for full platelet function. Once platelet GPCRs are activated by their own agonists, they initiate various intracellular signaling pathways that include activation of phospholipase C (PLC), protein kinase C (PKC), and phosphoinositide (PI)-3 kinase. Both calcium and PKC contribute to activation of the small G protein, Rap1b, which, through interactions with the Rap1-GTP interacting adapter molecule (RIAM) and talin, are important for binding of fibrinogen and other ligands to integrin GPIIb/IIIa (αIIbβ), an essential facilitator of platelet aggregation (Smyth et al. 2009).

**Platelets and Environmental Effects**

There is an accumulating body of evidence highlighting a relationship between external environmental effects and diseases including cardiovascular diseases such as myocardial infarction (Peters et al. 2004). Environmental effects can include behavioral factors, lipid and sodium content of the diet, workload, and pollutant exposure, and more importantly smoking/tobacco use (Hammerschmidt 1982). One important mechanism that has been identified involves increased susceptibility to the formation of thrombi (increased risk of thrombosis due to hyperactive platelets) in diseased arteries, which ultimately leads to disease conditions such as myocardial infarction and stroke. It is noteworthy that the initiating events in thrombosis formation

imperatively involve platelet adherence, activation, and aggregation. To this end, thrombosis is one of the main mechanisms of smoke-induced cardiovascular events, and while cigarette smoking has been declining, usage of other forms of tobacco, including hookah, has been on the rise (Jukema, Bagnasco, and Jukema 2014a) .

**Platelets as a Target for Therapy**

The cardinal role played by platelets in the process of thrombosis provides a strong rationale for blocking their function in thrombotic disease states such as in coronary heart disease (CHD). Following adhesion at the site of exposed subendothelial matrix in the presence of shear force, platelets get activated and release their granule contents such as ADP (dense granules) and generate arachidonic acid from membrane phospholipids via the cyclooxygenase-1 (COX-1)/thromboxane synthase pathway and thrombin through the coagulation pathway on the surface of activated platelets. ADP, thromboxane $A_2$, and thrombin act on their G-protein-coupled receptors: P2Y1 & P2Y12, TPR, as well as PAR1 and PAR4, respectively, and a cascade of intracellular signaling events culminate in the activation of the GPIIb/IIIa (aka αIIbβ3) receptor that then binds to fibrinogen to mediate platelet aggregation. Studies showed that the contributions of these upstream receptors to thrombosis is very important. Therefore, targeting these receptors (COX1 inhibitors, P2Y12 receptors blockers, and PAR1 receptors blockers) is one of the most current effective means to inhibit platelet function and to combat cardiovascular (CV) events in high-risk patients. Nonetheless, these current antiplatelet agents come with caveats, that is, many of these therapies are associated with dangerous side effects such as bleeding. Furthermore, some of these treatments are associated with a high rate of resistance as well as failure (Cedillo-Salazar et al. 2019, Paven et al. 2019). To this end, there is an urgent need for more specific targets to

inhibit platelet function and at the same time avoid the major side effects that are associated with the current available antiplatelet agents.

# Chapter II: The Effect of Waterpipe/Hookah on Platelet Function

## Introduction

Hookah also known as waterpipe, narghile, argileh, shisha, hubble-bubble, goza, borry, qaylan, chica and mada'a (Figure 1.1) is a tobacco pipe with a long yet flexible tube that draws the smoke through water contained in a bowl (Bou Fakhreddine, Kanj, and Kanj 2014). Even though hookah use in the western world is a recent trend, it has existed for a millennium; emerging in the North-Western provinces of India, spreading to Iran, the Arab world, Turkey and now gaining popularity in the US and Europe (Jukema, Bagnasco, and Jukema 2014a).

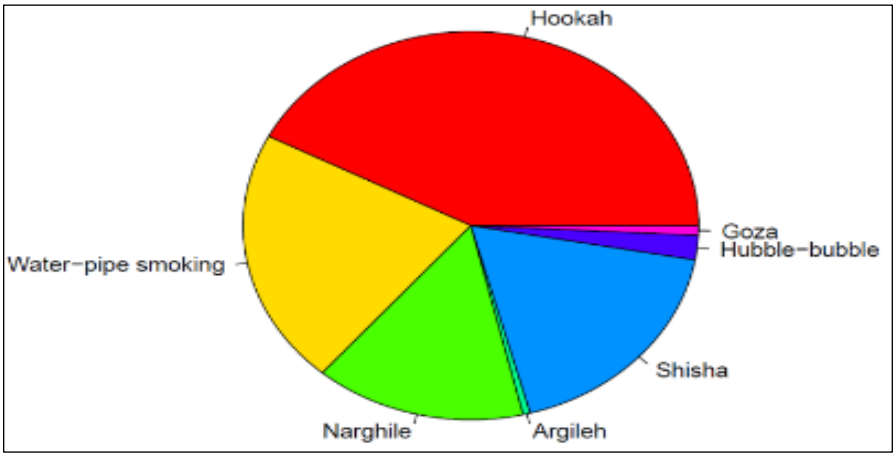



Figure 1.1**: PubMed search results for the different names of hookah used in the literatures.** Hookah is the most common used term followed by water-pipe and shisha.

## Apparatus Description

The hookah or hookah apparatus is composed of an upper and lower compartment connected by a pipe (Figure 1.2). Briefly, the top consists of a bowl where tobacco or molasses are placed then covered with perforated aluminum foil above which burning charcoal is placed. On the bottom of the apparatus resides a water jar covered by a gasket, protruding a hose and a release valve (used for clearing out stagnate smoke) (Bou Fakhreddine, Kanj, and Kanj 2014, Jukema,

Bagnasco, and Jukema 2014a, Nakkash and Khalil 2010a). A detailed description of hookah components is provided in *Table 1.1: Hookah apparatus components and their use.*

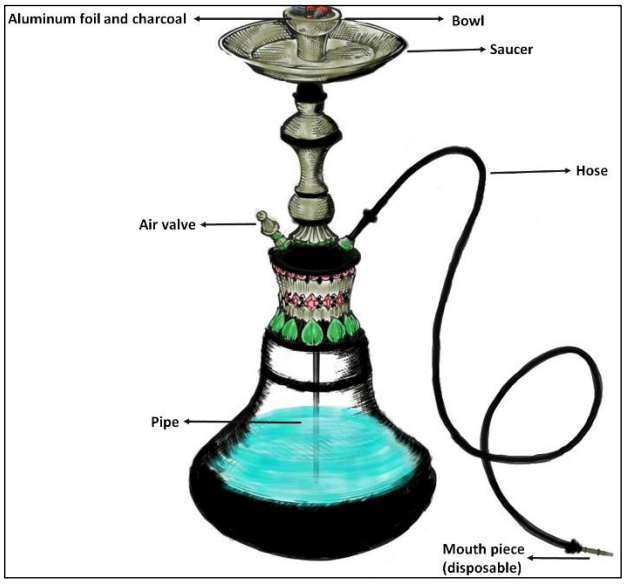



**Figure 1.2: A typical hookah apparatus.**

Table 1.1: Hookah apparatus components and their use.

| **Component** | **Usage/ properties** |
|---|---|
| ***Apparatus (Chaouachi 2011, Bou Fakhreddine, Kanj, and Kanj 2014, Jukema, Bagnasco, and Jukema 2014a, Nakkash and Khalil 2010a)*** | |
| Water Jar (base) | Glass container that is filled with water, however, other liquids might be used (milk, fruit juices or even alcohol) |
| Pipe (body, stem) | A metallic tube extending from the bowl to the base (partially immersed in water) |
| Bowl (head) | Carries (10-20g) of tobacco |
| Saucer | Holds charcoal ashes |
| ***Accessories (Nakkash and Khalil 2010a)*** | |

| | |
|---|---|
| Filter | A device that can be placed on the tip of the hose, marketed with claims that it reduces exposure to nicotine and tar. |
| Mouthpiece (extension) | A short plastic tip fitted into the hose as an extension to it. |
| Aluminum foil | Used as a membrane between tobacco (beneath it) and the charcoal (above it). |
| ***Consumables (Nakkash and Khalil 2010a, Chaouachi 2011)*** | |
| Charcoal | A special type of charcoal round pellets that are easily lit (Nakkash and Khalil 2010b) (made from wood, coconut shell or other material). |
| Tobacco | Marketed in two forms (moassel: fruit-flavored tobacco, pliable and moist) and (ajami: unflavored tobacco).<br>(Steam stones: heat-treated materials soaked with glycerin) are new "healthy" tobacco alternatives are released. (Lee, Mukherjea, and Grana 2013) |

Even though hookah retains some of its features globally (presence of liquid through which smoke passes), there is immense variability in the consumables, sizes, and materials used to manufacture apparatus parts; due to personal preferences and cultural/regional differences (Chaouachi 2011, Regulation. 2015). It is noteworthy that manufacturing material variability may influence the levels of smoke/chemicals exposure. Indeed one study reported that differences in pipe material affected the levels of carbon monoxide(CO) exposure, correlating the non-porous plastic hose with higher yields of CO compared to the more porous leather hose (Saleh and Shihadeh 2008). The same theory could apply to yields of other chemicals, especially nicotine.

Hence, hookah effects may be under/overestimated in some studies, so future research should take into consideration such variations to make results more relevant.

**Hookah Tobacco**

There are three commonly used types of hookah tobacco, Mouassal, Jurak, and Tumbak, each contains different ingredients. In brief, Mouassal which is an Arabic translation for "honeyed" contains 30% tobacco, and around 70% honey/sugarcane as well as glycerol and flavors(Khater et al. 2008). Hadidi et al (2004) estimated that the nicotine content of Mouassal is about 3.4mg/g. Jurak on the other hand, contains tobacco, sugarcane, and around 20% other spices and dried fruits (Hadidi and Mohammed 2004). Jurak is commonly used in the Middle East and Gulf region. Finally, Tumbak, which is used mainly in Asia, is a pure form of unflavored tobacco leaves (Ajami) smoked with charcoal.

**Hookah Tobacco Flavors**

In the US, there are different flavors used in hookah tobacco with the most popular being the fruit flavors (Smith-Simone et al. 2008). Similarly, among university students, fruit-flavored tobacco was preferred to unflavored ones (Salloum, Maziak, et al. 2015). Among US women, candy/sweet and menthol are the second and third preferred flavors respectively, with fruit flavors still number one choice (Scott-Sheldon and Stroud 2018). Other flavors include chocolate, clove/spice, alcohol, and other beverages (Smith-Simone et al. 2008). This suggests that flavored tobacco plays a major role as a "motivator" for using a hookah, which provides the user with pleasant taste and smell.

**Hookah Preparation and Mechanism of Operation**

The user or the person preparing the hookah (Figure 1.2) starts by loading the tobacco into the bowl before wrapping the head with aluminum foil and then perforating the foil by using a

screen pincher or toothpick. After that, the "ignited" charcoal is placed on the top perforated foil to initiate the tobacco heating process (Kim et al. 2016). During inhalation, charcoal heated air passes through the pierced aluminum foil and through the tobacco down the pipe and towards the water. After "bubbling" through the water, the cooled smoke reaches the surface and is drawn through the hose and is inhaled (Gatrad, Gatrad, and Sheikh 2007, Nakkash and Khalil 2010a). Taken together, hookah smoking seems to have a complex puffing behavior when compared to conventional cigarette smoking.

**Puffing Topography**

Both cigarette and hookah-smoking topography serve as an indirect measure of smoke and chemical exposure (Kim et al. 2016, Lee et al. 2003). In comparison to cigarettes, hookah-puffing is more variable including total puffing time, number of puffs, and total smoke inhaled, all being affected by nicotine content of tobacco, presence of flavors, personal preferences and the social setting of the vaping session (Blank et al. 2011, Kim et al. 2016, Cobb et al. 2015). Regarding total puffing time, hookah use takes significantly longer periods (30-90 minutes/session) (Kadhum et al. 2014, Cobb et al. 2012, Shihadeh 2003a, Maziak et al. 2004, Kim et al. 2016) in comparison to cigarette smoking (averages 5-6 minutes) (Djordjevic, Stellman, and Zang 2000, Kim et al. 2016). Furthermore, the number of puffs, mean puff duration, puff volume and inter-puff intervals were higher in hookah (Blank et al. 2014, Eissenberg and Shihadeh 2009, Anand et al. 2013), in contrast to conventional smoking (Krebs et al. 2016, Matsumoto et al. 2013). The longer sessions of hookah smoking could explain the increase in number of puffs/sessions. Also, the "humid" nature of the hookah smoke makes it more pleasant than the dry cigarette smoke and facilitates higher volume uptake (Cobb et al. 2015, Shihadeh and Saleh 2005, Blank et al. 2011). Importantly, this higher humidity of the smoke and its cooled-down nature facilitates deeper inhalation potentially

increasing the side effects of using hookah (Jukema, Bagnasco, and Jukema 2014a). Given the behavioral complexity of hookah use/smoking, further examination of smoking patterns is warranted in order to accurately estimate users' exposure to harmful chemicals.

**Reasons and Prevalence of Hookah Use**

As mentioned before, hookah became widely popular, with its use accelerating rapidly especially among youth and women (Palamar et al. 2014, Shearston et al. 2016). Thus, understanding the reasons/ patterns of use will aid in developing strategies to better control hookah use. Although there are ample justifications in the literature for hookah use, in this section we will include the most commonly reported, in addition to the prevalence of hookah.

**Reasons for Use**

Many factors seem to have promoted hookahs' spread/use, including but are not limited to perception of "no/less harm" of hookah, social acceptance/less restrictions, accessibility, use of flavored aromatic tobacco, curiosity, peer pressure, fashion, higher socioeconomic status and need for amusement (Amin et al. 2012, Martinasek, McDermott, and Martini 2011, Kelishadi et al. 2007, Baheiraei et al. 2015, Wong et al. 2016, Palamar et al. 2014, Ward et al. 2007, Giuliani et al. 2008). One factor that drastically contributed to the increased hookah use (similarly to e-cigarette use) is the misperception about the health risks. Majority of users believe in the "no or less harm" of hookah compared to cigarettes; this particular belief could be connected with the myths of intermittent use of hookah that reduces harm compared to constant use of cigarettes (Maziak, Eissenberg, and Ward 2005), the passage of smoke through water would filter it, and "the less" addictive nature of hookah (Jukema, Bagnasco, and Jukema 2014a). Some users argue that they do not inhale the smoke (keep it in the mouth cavity), therefore protecting themselves from nicotine absorption/addictive effects. However, nicotine could be easily absorbed through

the mucosal lining of the oral cavity (Jukema, Bagnasco, and Jukema 2014a). Furthermore, receiving the "positive" attributes of hookah such as socializing, relaxing and the good taste/smell of the smoke seems to encourage and maintain hookah use (Smith-Simone et al. 2008, Primack et al. 2008). To this end, a recent study conducted on social media (Twitter) found that social events and flavors were among the common contexts and experiences associated with Twitter discussions about hookah (2017-2018) (Allem et al. 2018). Finally, one study demonstrated that participants preferred flavored hookah because the "sweet" flavored smoke smell is not viewed as offensive as cigarette smoke (Giuliani et al. 2008), which supports the conclusion that flavor plays an important role in promoting hookah use. Thus clearly, there should be more emphasis on research studies that examine hookah health risks, which would inform campaigns for educating the public on the myths and the negative health effects associated with hookah use.

**Prevalence**

Worldwide, it is estimated that 100 million people use hookah on a daily bases (Ward et al. 2005). Back in 2011 "current hookah use" among adults age >18 years was 15% in Lebanon, 9-12% in Syria 4-12% in Arabic gulf countries and 6% in Pakistan (Akl et al. 2011), and 30% in Jordan (Khabour, Alzoubi, Eissenberg, et al. 2012) whereas in Iran it was found that more nonsmokers transition to tobacco use including waterpipe/hookah (Khosravi et al. 2018). Comparable to those levels, US adults "current use- smoking waterpipe on at least 1 day within the past 30 days" was 9.8% and "ever use- smoking waterpipe at any point in lifetime" was 1.5%

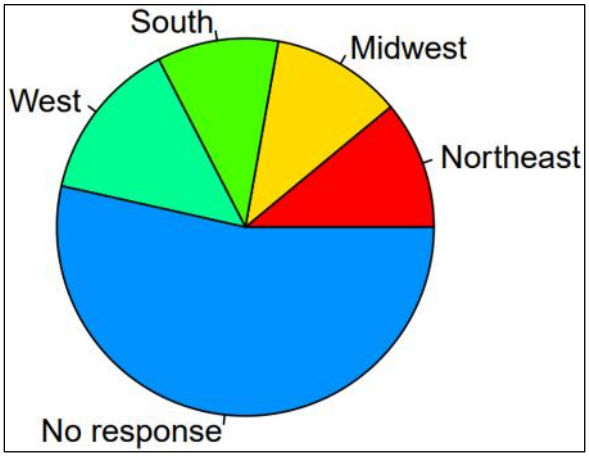



Figure 1.3**: Distribution of hookah "ever use" in the United States.** From the National Adult Tobacco Survey (NATS) 2012-2013 (n = 60192)

between 2009-2010 (Salloum, Thrasher, et al. 2015) reaching levels of 12.3% and 3.3% respectively by 2012-2013, reflecting a gross increase within the US population (Park et al. 2017b). Furthermore, while the majority of US hookah users are also tobacco smokers, a significant portion of hookah users are non-smokers (Ward et al. 2007, Primack et al. 2008). In this connection, differences between rural and urban US in smoking hookah was also examined, and the results illustrated more prevalence of hookah use in urban areas in comparison to rural areas (Roberts et al. 2017). This difference could be attributed to the sociocultural and economic factors linked to living in urban areas. Besides that, the distribution among the states/regions was found to be variable (Park et al. 2017a) (Figure 1.3), with west states having higher prevalence compared to south states; in particular, five states, namely Arizona, California, Colorado, the District of Columbia and Nevada had high rates of both current hookah use ($\geq 5\%$) and ever use ($\geq 15\%$)(Park et al. 2017b).

It is difficult to determine what might be the cause behind this increase in the west, but potentially it might derive-in part- from the higher population of Arab Americans within these regions. This notion is supported by Arab Americans have the highest rates among all racial/ethnic groups of adults identifying themselves as non-Hispanic "Other"(Salloum, Thrasher, et al. 2015). Regardless, conducting research on hookah environment characteristics such as the number of hookah bars, methods of advertising, and social behaviors could serve as the first step in further understanding the increasing prevalence and popularity of hookah.

Hookah use among the US youth population in schools was under scrutiny in many studies. For instance, one study surveyed a representative sample of students $6^{th}$- $12^{th}$ grade for hookah use, and the results showed that 10.5% reported smoking hookah (Agaku et al. 2018). Another study documented that hookah "ever use" among middle and high school students in the US included

6.8-15% of the students (Martinasek, McDermott, and Martini 2011). These results are remarkable as they show that as young as sixth grade (vulnerable population) can be a user of hookah, and potentially exposed to all associated health risks. According to the same study, household hookah users and easy access are of the main motivation factors to use hookah in such a young age (Agaku et al. 2018). Interestingly, Arab Americans had a higher percentage of both "current and ever use" compared to non-Arabs, which indicates a strong influence of cultural background on hookah use (Weglicki et al. 2008).

College students' prevalence of hookah use was 9.6% for "current use" and 22.9% for "ever use" between 2008-2009, whereas it increased to 28.4% for "past year" use and 46.4% for "ever use" in 2013 (Barnett et al. 2013). Such an alarming increase may be in part due to the belief that hookah use is less harmful/addictive with higher social approval in comparison to tobacco (Heinz et al. 2013, Noonan and Patrick 2013). Notably, this belief is the major reason many adults use hookah, in addition viewing it as a good way of socializing, and the belief that it helps quitting cigarettes, as well as being relatively cheaper than smoking cigarettes (Wong et al. 2016). Another drastic and more concerning increase of 5.3% from 2011 to 2014 in hookah popularity was reported among adolescents in US. This is especially troublesome as adolescents continue to be exposed to harmful tobacco product constituents, in particular, nicotine that might interfere with brain development, cause addiction, and might lead to sustained future tobacco use (Arrazola et al. 2015). Another recent representative sample of young adults aged 15-24 years old, revealed that hookah use was 14.7% among males and 10.2% among females (Li et al. 2018). This increase in use could be attributed to the perception of fewer negative consequences of hookah smoking compared with cigarette smoking and the social norms regarding its acceptability among this population (Holtzman, Babinski, and Merlo 2013, Montgomery et al. 2015, Berg et al. 2015).

Importantly, developing new perhaps "more rigid" policies to regulate hookah use, is not only needed in youth but must also expand to control use during pregnancy. This is rather a public health priority given hookah's prevalence (12.4%) (Kurti et al. 2017), coupled with the relatively "high" passive exposure to hookah smoke (32.8%) during gestation (Azab et al. 2013); which in turn leads to the involuntary exposure of innocent fetuses to hookah, and subjecting them to hookah's potential harmful effects (Kahr et al. 2015).

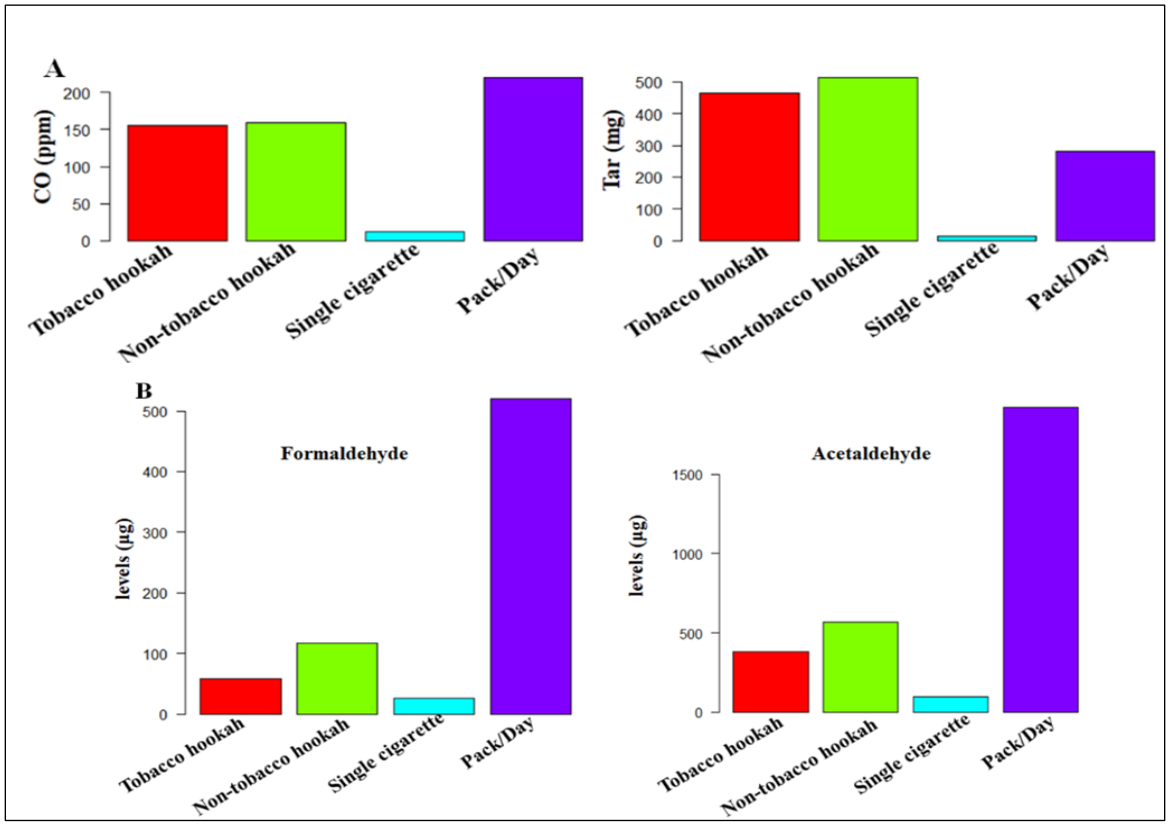
Regardless of the variability in hookah prevalence amongst the various populations, clearly, there is an overall drastic increase in its use over a short period. Longitudinal studies should further help in understanding and evaluating use trends, sociodemographic characteristics, health risks in various populations being exposed to/using hookah, which would ultimately shape new "more strict" policies, especially those governing the use of highly vulnerable populations, including pregnant females, and youth.

**Toxicological Profile**

Even though hookah has been present for a millennium, far less studies have examined its chemical Constituents/air quality relative to cigarettes. With tobacco being the main source of smoke in both hookah and cigarettes, hookah users are exposed to many of the same toxic compounds/by products as cigarette users but at dramatically higher levels, which might, in fact, produce worsened health effects in users (Eissenberg and Shihadeh 2009). Consequently, it is important to evaluate the major compounds expelled from hookah vape, in order to aid in evaluating both acute and chronic health outcomes.

Several toxicants have been found in mainstream hookah smoke including nicotine (Shihadeh and Saleh 2005, Schubert et al. 2011, Shihadeh 2003b, Jacob et al. 2011, Shihadeh et al. 2012), carbon monoxide (Jacob et al. 2011, Shihadeh and Saleh 2005), carcinogenic polycyclic

aromatic hydrocarbons (PAHs) (Shihadeh and Saleh 2005, Jacob et al. 2011, Schubert et al. 2011, Sepetdjian, Shihadeh, and Saliba 2008b, Shihadeh et al. 2012), aromatic amines (Jacob et al. 2011), aldehydes (Shihadeh et al. 2012, Al Rashidi, Shihadeh, and Saliba 2008), furanic and phenolic compounds (Sepetdjian et al. 2013, Schubert et al. 2012), tar (Schubert et al. 2011, Shihadeh 2003a), particulate matter (Monn et al. 2007) heavy metals (Shihadeh 2003a) and ammonia (Baker and Dixon 2006). It is noteworthy that the amounts of these toxicants might be higher/lower in hookah compared with cigarette smoke (per cigarette/ and per pack/day) due to different heating processes and charcoal combustion (Chaouachi 2009, Shihadeh et al. 2014, Calafat et al. 2004, Moldoveanu, Coleman, and Wilkins 2008) (Figure 1.4).



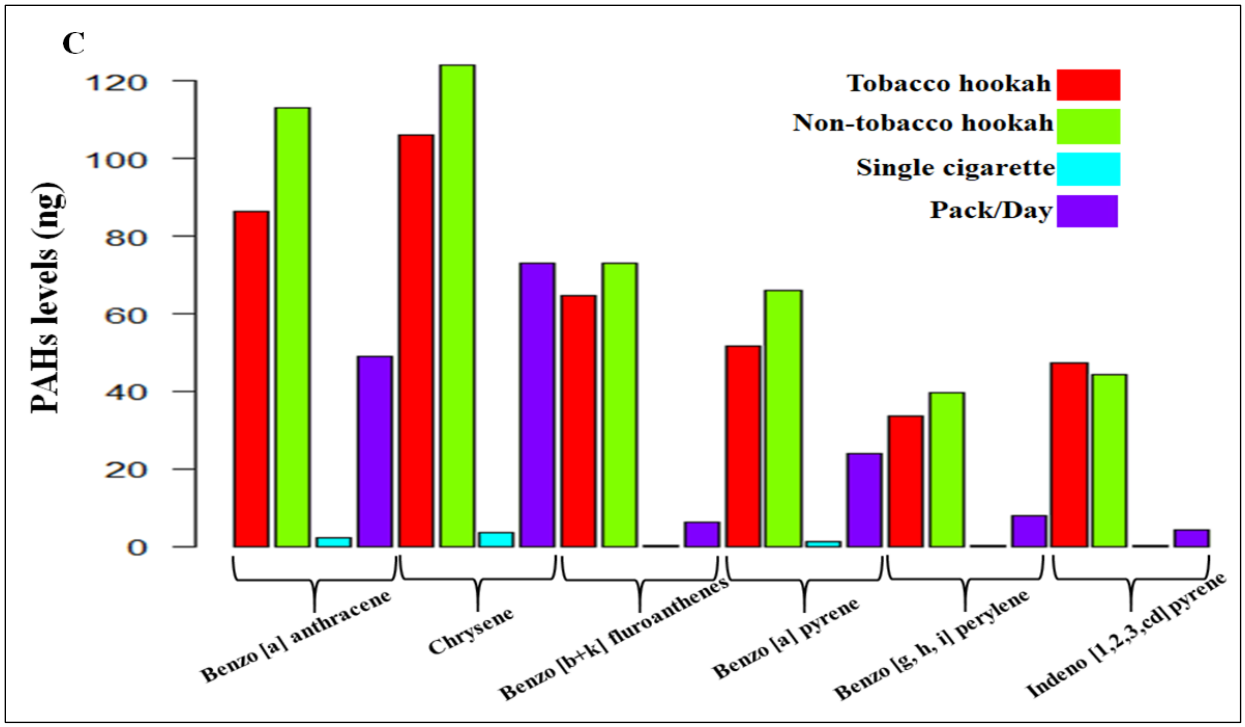



Figure 1.4**: Comparison of the levels of some toxicants expelled in both hookah and tobacco smoke**. (A) Levels of the toxicants tar, CO. (B) carbonyl compounds (formaldehyde and acetaldehyde), and (C) certain PAHs are indicated. Levels of toxicants in pack/day are extrapolated by multiplying the levels in one cigarette by 20 (Chaouachi 2009, Shihadeh et al. 2014, Calafat et al. 2004, Moldoveanu, Coleman, and Wilkins 2008).

Nicotine- the major source of tobacco addiction- content in hookah is extremely variable as it depends on the type of tobacco used. Consequently, the amount up-taken by the user depends on hookah use characteristics that are adjusted depending on nicotine levels in tobacco used in order to deliver desired doses (Shihadeh 2003b). Similar to cigarette smoking, plasma nicotine levels were found to be increased in hookah users, indicating systematic delivery of nicotine. However, these levels were much higher compared to cigarette users, which could be explained by longer "hookah" sessions with a higher puff number/volume (Eissenberg and Shihadeh 2009, Cobb et al. 2011). Likewise, plasma carboxyhemoglobin levels in hookah users exceeded those of cigarette smokers' levels (Schubert et al. 2011), indicating the presence/inhalation of carbon monoxide (CO) during hookah use. This is because CO displaces $O_2$ from hemoglobin forming

carboxyhemoglobin (CO affinity for hemoglobin is 200 times that of $O_2$), and shifting the oxygen dissociation curve to the left, thereby causing hypoxia and impairment of cellular respiration (Weaver 2009). Notably, hookah use was linked to several cases of CO poisoning (Cavus et al. 2010, Lim, Lim, and Seow 2009, Türkmen et al. 2011, La Fauci et al. 2012, von Rappard, Schonenberger, and Barlocher 2014). Furthermore, NNAL- a metabolite formed after 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) enters the body and a well-known carcinogen - urinary levels increased markedly after hookah use, indicating the presence of tobacco-specific nitrosamines in hookah smoke(Jacob et al. 2011, Radwan et al. 2013). Other well-known carcinogens/ potential carcinogens are polycyclic aromatic hydrocarbons (PAHs) and they have been quantified in hookah smoke, in particular, 16 of these compounds have been found (Sepetdjian, Shihadeh, and Saliba 2008b). Moreover, in contrast to single cigarette smoking, nicotine-free dry particulate matter (TAR) from single 45 minutes hookah session reached a level of 802 mg, which represents 36.5 folds higher than that in a cigarette. This should in turn diminish marketing fads of hookah containing 0% tar (Jawad, Khaki, and Hamilton 2012). Of note, levels of tar delivered vary from session to session, reaching up to 100 folds in some cases of longer hookah smoking sessions (Jacob et al. 2013).

Importantly, inhaled volumes are concerning because they deliver high amounts of hazardous chemicals/session compared to cigarettes. For instance; aldehydes, such as acrolein, induce cardiopulmonary toxicity (Conklin 2016, Kassem, Kassem, Liles, Zarth, et al. 2018), are potentially carcinogenic (Tang et al. 2011, Feng et al. 2006) and are prothrombotic (Sithu et al. 2010b). Furthermore, the PAHs are carcinogenic (Abdel-Shafy and Mansour 2016), whereas carbon monoxide induces cardiovascular disease (Zevin et al. 2001), and nicotine is known for its addictive nature (Benowitz 2010). As for ammonia, which is a strong respiratory irritant, its levels

should be measured as part of the assessment of the hookah toxicant profile, and this could be achieved using a simple colorimetric method as was recently described for determining ammonia levels in tobacco fillers and sidestream smoke in different tobacco brands (Inaba, Uchiyama, and Kunugita 2018).

Based on the aforementioned considerations, hookah use poses as many or even higher risks for the smoker as cigarette smoking. Besides tar, hookah bears additional risks such as an uptake of addictive and carcinogenic/ potentially carcinogenic chemicals, which stands in contrast to the "massive" advertisement of them as a "healthy" smoking product.

**Air Quality and Passive Exposure**

Ambient concentrations of particulate matter are often used to assess pollution levels from tobacco smoke (Fiala, Morris, and Pawlak 2012). Cigarette smoking expels high levels of particulate matter in bars, exposing both customers and employees passively to hazardous levels of pollutants (Centers for Disease and Prevention 2004). In a similar fashion, the examined air quality in hookah lounges ranged from "unhealthy" to "hazardous" by the Environmental Protection Agency (EPA), containing high concentrations of particulate matter (Fiala, Morris, and Pawlak 2012, Zhou et al. 2015, Zhou et al. 2016). Such air quality poses health risks, especially among those with existing pulmonary and cardiovascular disease, and presents potential health hazards for workers who can be exposed to secondhand hookah/smoke on a daily basis, and for prolonged periods of time (Fiala, Morris, and Pawlak 2012). As expected, low air quality was reported in houses of hookah users, and interestingly toxicant levels were greater than those in cigarette smoking homes, yet less than those in lounges/bars (Figure 1.5- (Weitzman et al. 2016, Zhou et al. 2015, Fiala, Morris, and Pawlak 2012)); which is probably due to lower numbers of hookah apparatuses used (Weitzman et al. 2016). Nonetheless, such low air quality exposes non-

users within the household to hazardous materials, and puts them under increased risk of disease, especially if they are highly vulnerable (with chronic disease, children and pregnant women). Consequently, there is a marked need for further research, policies and better air quality monitoring to improve the indoor air quality in order to reduce passive exposure and its negative health consequences.

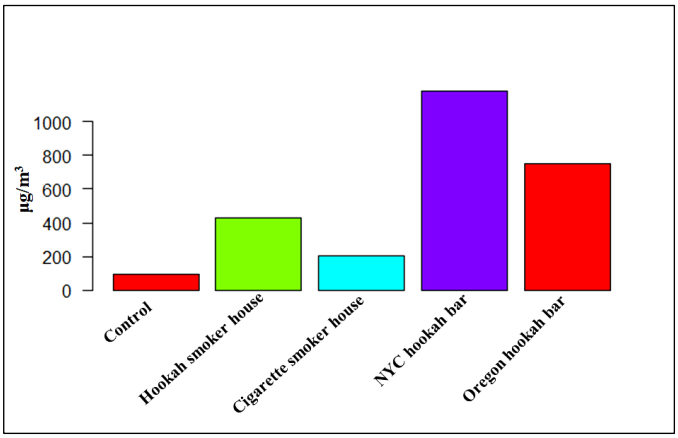



Figure 1.5: **Comparison between PM2.5 levels in houses of hookah users, cigarette users and bars in two states.** NYC hookah bars (n=8). Oregon hookah bars (n=10) (Weitzman et al. 2016, Zhou et al. 2015, Fiala, Morris, and Pawlak 2012).

## Hookah Health Hazards

While hookah users and those in close proximity are exposed to many of the potentially dangerous toxicants at one time, the health risks associated with its use continue to be under debate. This is-in part- attributed to the fact that the composition of tobacco smoke in hookah and its puffing patterns are variable and not well standardized. Nonetheless, several studies have provided evidence of health impairments that are associated with hookah use.

Theoretically, sharing the mouthpiece during hookah group smoking can be a probable source of transmission of pathogens such as viruses, bacteria, and fungi. For instance, a study reported a potential risk for transmission of communicable diseases such as hepatitis C when sharing the mouthpiece between users with bleeding gum (Habib et al. 2001). Also, the “non-

hygienic" conditions of the hose and water in the hookah apparatus could also increase mycobacteria growth, which can result in the spreading/transmission of tuberculosis (Urkin, Ochaion, and Peleg 2006, Blachman-Braun et al. 2014). Other studies have also linked hookah to transmission of Helicobacter pylori (the main cause of peptic ulcer) and Aspergillus spores (cause of pneumonia in immunocompromised patients) (El Barrawy, Morad, and Gaber 1997, Szyper-Kravitz et al. 2001). Moreover, 48 bacterial isolates were detected from hookah hoses, and among them were virulent as well as antibiotics resistant strains (Masadeh et al. 2015). Furthermore, using hookah was linked to developing periodontal diseases in a similar magnitude to cigarettes (Natto 2005) as well as a documented alteration in oral microbial flora (Shakhatreh et al. 2018).

Similar to cigarette smoking, hookah use is also linked to a harmful impact on the pulmonary system. Thus, hookah users complain of symptoms such as wheezing, cough, sputum, and shortness of breath (Boskabady et al. 2012, Boskabady et al. 2014, Strulovici-Barel et al. 2016). Furthermore, hookah significantly decreases pulmonary function parameters, including FEV1, FEV1/FVC ratio, and FEF, as well as the levels of FeNO (Meo et al. 2014, Raad et al. 2011, Hakim et al. 2011). FeNO is an essential marker of eosinophilic airway inflammation, and reduction in its levels may be due to the rapid conversion of nitric oxide to peroxynitrite by reactive oxygen and nitrogen species or down-regulation of nitric oxide synthase (Barua et al. 2003, Hoyt et al. 2003). Also, hookah users had lower lung diffusing capacity and elevated levels of apoptotic endothelial cell microparticles (Strulovici-Barel et al. 2016). Hookah exposure induced a significant elevation of macrophages, lymphocytes and neutrophils in broncho-alveolar lavage fluid and altered the levels of several cytokines. Thus, the levels of the pro-inflammatory cytokines TNFα, IL-1 β, IL-6, IL-12, and IL-13 were elevated, whereas the levels of the anti-inflammatory cytokine IL-10 were reduced, in the lungs of exposed mice (Khabour, Alzoubi, Al-Sawalha, et al.

2018, Al-Sawalha, Migdadi, et al. 2017). It also increased catalase activity in the lung and resulted in changes in the level and mRNA of major matrix metalloproteinase (MMP-1, -9, and -12), confirming pulmonary damage associated with hookah use (Khabour, Alzoubi, et al. 2012a, Khabour et al. 2015). Moreover, chronic (4 months) exposure to hookah smoke in mice resulted in significant increases in alveolar destructive index and mean linear intercept contributing to the chronic obstructive pulmonary disease picture in these animals (Alzoubi et al. 2018).

Importantly, hookah hazards are not limited only to oral/pulmonary systems. To this end, in a population-based study, hookah use was associated with metabolic syndrome development. Thus, hookah users had a significantly higher incidence of hypertriglyceridemia, hyperglycemia, as well as hypertension and abdominal obesity, which was observed after controlling for age, sex, social class and area of residence (Shafique et al. 2012). All of these "disorders" increase the risk of metabolic syndrome development, which is a major risk factor for developing thrombosis (Kaur 2014).

Unfortunately and as stated before, the number of hookah users among the vulnerable populations of pregnant females and adolescents is increasing. In fact, pregnant females still use hookah during pregnancy, regardless of its reported hazards. While it may vary based on levels of use/exposure, a reduction of weight of the newborn (at least 100 gm) in females using hookah once/day during pregnancy was evident. Moreover, the risk of delivering low birth babies tripled, in addition to reported neonatal respiratory distress that is linked to hookah use during first trimester (Nuwayhid et al. 1998, Tamim et al. 2008). In addition to reduction in birth weight, it was reported that hookah smoking during pregnancy contributes to a significant reduction in newborns' other anthropometric measurements such as mean newborn length, and mean newborn head circumference (Al-Sheyab et al. 2016). In a rat exposure model, hookah smoke exposure was

shown to be associated with low birth weight, increased neonatal death rate, and lower growth rate among offspring (Khabour et al. 2016). Additionally, prenatal exposure to hookah smoke in a murine model of asthma in adult mice offspring also induced airway inflammation and adversely affected lung function (Al-Sawalha, Al-Bo'ul, et al. 2017). In utero exposure to hookah tobacco smoke in rats resulted in impaired memory and decreased Brain-Derived Neutrophil Factor (BDNF) in the hippocampus of adult male offspring rats (Al-Sawalha et al. 2018).

A study of hookah use among $7^{th}$ -$10^{th}$ grade students indicated that it may impair adolescents' brain development, given that it reduces the levels of the BDNF (Yeom et al. 2016), which is essential for cognition and behavior (Yeom et al. 2016). A relatively recent study also reported a reduction in BDNF serum levels in students reflecting a possibility of systematic adverse health alterations in adolescence, coupled with behavioral changes such as low attention and aggression; (Alomari et al. 2018b) . Moreover, hookah tobacco smoke exposure in rats induced short- and long-term spatial memory impairment (Alqudah et al. 2018), which was associated with reduced hippocampal levels of major oxidative stress biomarkers and oxidative capacity enzymes (Alzoubi et al. 2015, Alzoubi et al. 2019).

With respect to the carcinogenicity of hookah, it was reported that carcino-embryonic antigen (CEA) levels were higher in hookah smokers, in comparison to non-smokers, yet not as high as in cigarette smokers (Sajid et al. 2007). Thus, prolonged or heavy use of hookah may induce a risk of tumor development, especially in the oral cavity and esophagus, which further argues against the notion that hookah has “no/less harm” if not inhaled “kept in mouth”. Indeed, the incidence of benign lesions of the vocal cords was linked to the presence of cysts in 4.8% of hookah users, which was similar to cigarette smokers (Hamdan et al. 2010). Furthermore, three case-control studies reported a link between the risk of esophageal cancer and hookah use, with

the risk increasing with cumulative use, higher frequency and the duration of use (Dar et al. 2012, Nasrollahzadeh et al. 2008, Malik et al. 2010). Additionally, using hookah was linked to an average of six folds higher risk of lung cancer (Lubin et al. 1992, Gupta et al. 2001, Aoun et al. 2013, Koul et al. 2011). Moreover, it was reported that hookah use may increase the risk of gastric cancer by three-fold, albeit the mechanism remains unknown (Sadjadi et al. 2014). In addition, hookah smoking was shown to be genotoxic, leading to DNA damage in lymphocytes, where the magnitude of its genotoxicity was higher than that induced by cigarette smoking (Khabour et al. 2011, Alsatari et al. 2012). Exposure to hookah smoking resulted in elevated plasma and saliva levels of toxic metals, namely, cadmium, copper and zinc (Khabour, Alzoubi, Al-Sheyab, et al. 2018), which could contribute to its long-term carcinogenicity. Finally, another study (analyzed data collected from 152 academic institutions; n=100,891 students) found a moderate association between hookah smoking and mental health variables, such as depression, anxiety, and addictive disorders, among college students (Primack et al. 2013). These findings provide evidence that hookah not only disrupts the "physical" health of the user but also their mental state.

It is noteworthy that many of the aforementioned studies had limitations, for example, no control over use of other forms of tobacco, lack of adjustments of the cofounding factors in some case-studies, as well as limited assessment of gender and age as cofounders. Nonetheless and taken together, there is sufficient evidence in support of the association of hookah use with negative human health outcomes. Considering the cardiovascular system sensitivity and its nonlinear dose-response/toxicity relationship with "smoke", the next section will review the literature with regard to the effects of hookah on the cardiovascular system (see Figure 1.6), both human and animal studies.

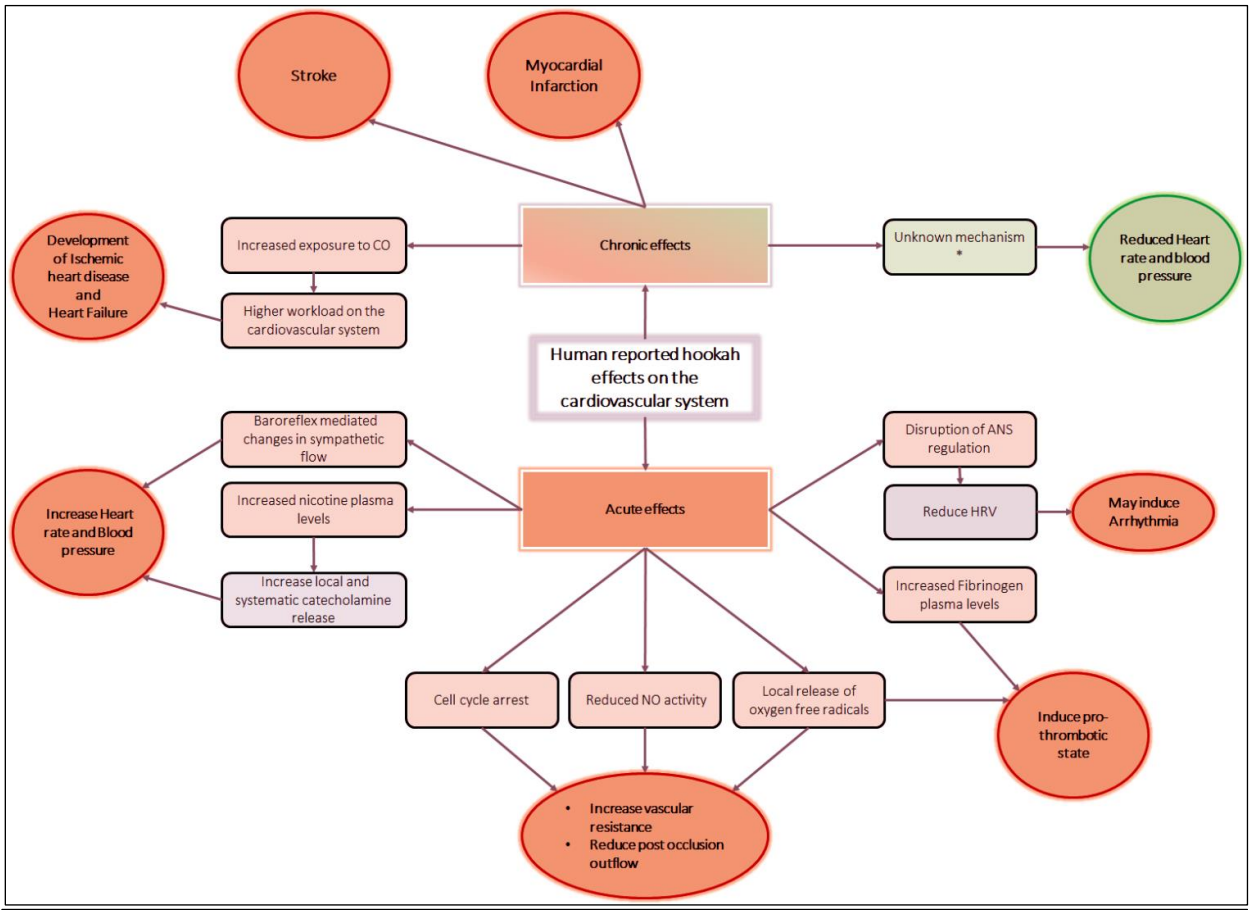



Figure 1.6**: Cardiovascular effects and their underlying mechanisms.** These data are compiled from what is reported in clinical studies**.** * Study performed in adolescents, the reduced heart rate and blood pressure may be linked to the "abstinence of vaping" for 12 hours prior to testing (Alomari and Al-Sheyab 2018).

## Cardiovascular Effects of Hookah Use

The detrimental (acute and chronic) effects of tobacco smoking on the cardiovascular system are well-established (Ambrose and Barua 2004b, Messner and Bernhard 2014, Benowitz , 2010, Bullen 2008, Michael Pittilo 2000). Thus, cigarette smoking predisposes to cardiovascular events and contributes significantly to cardiovascular related mortality and morbidity, being responsible for up to 30% of heart disease-related deaths in the United States each year (Benowitz , Qasim et al. 2017). Importantly, hookah has overlapping toxicant/chemical profile to conventional cigarettes. In light of that, it has been shown that hookah smoke effects on the cardiovascular system are comparable to those of conventional cigarettes. It is noteworthy that a

recent meta-analysis reported an odds ratio of association between hookah tobacco smoking and heart disease of 1.67 (95% CI = 1.25, 2.24) (Waziry et al. 2016), which supports the notion that hookah is indeed detrimental to the cardiovascular system.

## Clinical Studies

### *Acute cardiovascular effects*

Acute effects of conventional smoking, such as increased blood pressure, heart rate (HR) and vascular resistance have been known for decades (Jaquet, Shapiro, and Uijtdehaage 1994, Al-Safi 2005, Quillen et al. 1993, Rhee et al. 2007). As with tobacco smoking, the instantaneous effects of hookah use include higher systolic and mean arterial blood pressure, as well as elevated HR (Hakim et al. , Shaikh et al. 2008, Kadhum et al. 2014, Azar et al. 2016, Al-Kubati et al. 2006, Eissenberg and Shihadeh 2009, Hawari et al. 2013, Layoun et al. 2014). It is noteworthy that in one cohort study HR elevation has exceeded 50 bpm in 6.4% of participants, and reached higher than 200 bpm in 3.6% of participants after only a 30 minute-hookah session (OA-A. 2012). These effects have been attributed, in part, to the baroreflex mechanism impairment (Al-Kubati et al. 2006), or to elevated nicotine plasma level. The latter exhibits adrenergic effects that will enhance local and systemic catecholamine release (Cobb et al. 2012, Saafan 2005, Al-Kubati et al. , Al-Safi et al. 2009, Blank et al. 2011, Azar et al. 2016). Supporting the latter notion, mean post hookah-smoking heart rate elevation was doubled in participants using nicotine-containing hookah in comparison to nicotine-free hookah smokers (Shishani et al. 2014). Additionally, in a two double-blind study, “placebo” had no effect on HR, while hookah-smoking increased it significantly (Blank et al. 2011). Another study reported that changes in the cardiovascular central and peripheral components occur immediately after hookah smoking, and include increases in HR, blood pressure, and after occlusion vascular resistance, whereas after occlusion blood flow and

outflow were decreased (Alomari et al. 2014). The cardiovascular changes were shown to be exacerbated among individuals with low habitual physical activity and physical fitness levels (Alomari et al. 2015). More recently, it has been reported that adolescents smoking hookah had significantly lower vascular endothelium growth factor (VEGF) levels (Alomari et al. 2018a), which might adversely affect vascular growth and function in this population.

Acute use of hookah also induced changes in the peripheral vascular system in a similar fashion to cigarette smoking, such that it increased vascular resistance and reduced post occlusion blood flow. This could be linked to local release/synthesis of oxygen-derived free radicals, cell cycle arrest and a decrease in NO activity (Alomari et al. , Mayhan and Sharpe 1998, Diab, Abdelrahim, and Esmail 2015, Rammah et al. 2013). In a manner comparable to cigarette smoking, short-term hookah use significantly impaired flow-mediated dilation (FMD), which indicates endothelial dysfunction, but hookah was a weaker predictor for high-risk profile (Diab, Abdelrahim, and Esmail 2015). Furthermore, it was reported that short-term hookah use (both tobacco-based and tobacco-free products) disrupts the autonomic nervous system regulation on the cardiac cycle, thereby causing a reduction in HR variability; which- in turn- might aggravate the risk of coronary artery disease development (Cobb et al. 2012). Moreover, a significant increase in $TXB_2$ levels, a metabolite of the biologically active $TXA_2$ and an index of oxidative injury, was reported after a single hookah smoking session (Wolfram et al. 2003). This increase in $TXB_2$ levels would suggest an increase in platelet activity (Catella et al. 1986). Importantly, it has been shown that an increase in platelet activity plays a major role in the pathogenesis of acute myocardial infarction (MI) (Tousoulis et al. 2010) and acute stroke (Mulley et al. 1983, Cevik et al. 2013, McCabe et al. 2004). Therefore, it is not surprising to see a link between hookah smoking and acute MI in young adults (Abed, Eshah, and Moser 2018), and among patients undergoing cardiac

catheterization (Platt et al. 2017). However, no data exist yet on the association between hookah smoking and acute stroke (Hashmi, Khan, and Wasay 2013). Interestingly, and contrary to the hypothesis that hookah decreases myocardial blood flow because of the charcoal combustion nanoparticles (vasoconstrictor), a study found that hookah use acutely increased myocardial blood flow. This is thought to be due to cardiac β-adrenergic stimulation as a physiological response to increased myocardial work and oxygen demand (Nelson et al. 2016). In light of the aforementioned evidence, it is clear that even short-term use of hookah disrupts normal cardiovascular function, as repetitive short-term hookah exposure may be the triggering point of causal chain of reactions ultimately leading to the chronic effects. Nonetheless, more research should be done to evaluate hookah's effects, which will guide awareness campaigns regarding its negative health outcomes, including those that result from short-term use/exposure.

***Cardiovascular effects of chronic use***

With regard to the adverse cardiovascular effects associated with longer-term of hookah use, they are comparable to those associated with cigarette smoking. In this connection, a link between chronic use of hookah and coronary artery disease (CAD) development has been shown, with the frequency and duration of exposure being critical risk factors to CAD. In fact, individuals with more than 40 years of hookah smoking had three times more risk of having severe stenosis than nonsmokers (Sibai et al. 2014). Additionally, cardiovascular disease development such as ischemic heart disease (IHD) and heart failure have been associated with heavy hookah smoking (Islami et al. 2013). These outcomes could be explained by the continuous stress placed on the cardiovascular system as a result of exposure to high amounts of CO (Soule, Lipato, and Eissenberg 2015). Furthermore, death due to IHD was 1.96 folds in ever hookah smokers with a higher daily intensity of hookah smoking than never users (Wu et al. 2013). Hookah smoking was

also associated with severe CAD, which was dependent on the duration/frequency of hookah smoking (Selim, Fouad, and Ezzat 2013). In accordance with the latter data, a dose-response relationship between hookah-years and percent stenosis was also established(Sibai et al. 2014). Furthermore, the risk of MI and stroke death was significantly increased with hookah smoking. Finally, higher fibrinogen plasma levels were reported in long term hookah smokers (> 10 years), which elevates the incidence of pro-thrombotic/atherosclerotic events, (Seyyed Hashem Sezavar 2004) and might explain in part the higher risks of stroke and MI linked with chronic hookah smoking.

Notably and interestingly, a recent cross-sectional study aimed to examine the relationship between chronic hookah smoking and cardiovascular hemodynamics in adolescents found a reduction in both BP and HR of adolescent hookah smokers versus non-smokers, which is in contrast to previously reported results in adults. This might be explained by the "abstinence of" hookah smoking for 12 hours prior to testing, thereby reducing nicotine levels drastically in the system, which would impact neuro-hormonal regulation (reduced cortisol and sympathetic activity). Nonetheless, the exact mechanism underlying such an outcome is still unclear but warrants investigation (Alomari and Al-Sheyab 2018).

Together, hookah use has been associated with many cardiovascular effects that influence or contribute to the decline of the overall health status of members of our communities. Unfortunately, despite studies documenting cardiovascular disease risks associated with hookah, people continue to assume that it is safer than cigarettes, mainly due to being unaware of its negative health effects. Thus, hookah awareness/ control should be more robust and systematic, but more importantly, further studies need to be conducted to better understand its negative health consequences, and the mechanisms inducing such effects.

## Animal Studies

Acute and chronic exposures to hookah smoke, resulted in significant changes in kidney function biomarkers such as creatinine and blood urea nitrogen, in mice. This was associated with a reduction in antioxidant enzymes and biomarkers including superoxide dismutase for acute and chronic hookah smoke exposures, and catalase, glutathione peroxidase, and thiobarbituric acid reactive substances for chronic exposure (Rababa'h et al. 2016). Acute and chronic exposure of mice to hookah corroborates with the clinical findings that suggest cardiovascular dysfunction. To this end, short-term nose-only exposure to mainstream hookah for 5 consecutive days induced a significant decrease in platelet numbers and amplified *in vitro* platelet aggregation indicating a prothrombotic state (Nemmar, Yuvaraju, et al. 2015, Nemmar, Al-Salam, Beegam, et al. 2017). Furthermore, cardiac inflammation with an increase in reactive oxygen species (ROS) was observed, which consequently caused an elevation in heart glutathione (GSH; an antioxidant) concentrations. This seems to indicate that an initial adaptive response that counterbalances the potentially damaging activity of ROS (Nemmar, Yuvaraju, et al. 2015) is triggered. Interestingly, long-term nose-only exposure for one month caused a significant increase of ROS in the heart accompanied with decreased heart GSH concentrations in exposed mice, indicating depletion of the antioxidant, which increases heart tissue vulnerability to oxygen free radicals damage (Nemmar et al. 2013). The increased cardiac vulnerability may explain the increased systolic blood pressure reported after long-term use, which was not seen post short-term exposure (Nemmar, Yuvaraju, et al. 2015, Nemmar et al. 2013).

## The Gap in the Literature

Both clinical and animal studies have provided (although limited) evidence of a link between cardiovascular disease development and waterpipe/hookah (short and long-term use).

However, there remain some knowledge gaps; firstly, lack of well-designed studies and comprehensive meta-analysis addressing the association between hookah use and cardiovascular diseases. But more importantly, the pathophysiologic mechanisms that link waterpipe/hookah to cardiovascular adverse events are not fully understood. Therefore, there is a need for studies on the effects of waterpipe/hookah smoke exposure on cardiovascular diseases in the context of platelets. These types of studies are critical, knowing that thrombosis is one of the main mechanisms of smoke/tobacco-induced cardiovascular diseases, and the fact that platelets are a major player in the process of thrombosis. Further, waterpipe/hookah shares some of its toxic profile with cigarettes, which is established as a platelet activation modulator.

**The Research Question**

As a result of our literature research and the available evidence, we decided to ask the following question:

Does adult and *in utero* exposure to waterpipe/hookah smoke increase the risk of thrombosis and modulate platelet function?

**Hypothesis**

Adult and *in utero* exposure to waterpipe/hookah smoke modulates platelet function, thereby increasing the risk of thrombosis.

**Methods**

**Reagents and Materials**

Thrombin was purchased from Chronolog Corporation (Havertown, PA), whereas adenosine diphosphate (ADP), nicotine, and cotinine were from Sigma Aldrich (St Louis, MO). Fluorescein isothiocyanate—conjugated anti-P-selectin and fluorescein isothiocyanate—conjugated Annexin V were purchased from Cell Signaling Technology, Inc (Danvers, MA). The

phycoerythrin-conjugated αIIbβ3 antibody was obtained from Emfret analytics (Würzburg, Germany). Stir bars and other disposables were purchased from Chrono-Log Corporation (Havertown, PA). Akt (protein kinase B), phospho-Akt (Ser473), ERK (extracellular signal regulated kinases), and phospho-ERK antibodies were purchased from Cell signaling (Danvers, MA). The anti-PAR4 (protease-activated receptor 4) and anti-GPIIb-IIIa (αIIbβ3) antibodies were from Abcam (Cambridge, MA), whereas the anti-p85/PI3K (phosphoinositide 3-kinases) antibody was from Proteintech (Rosemont, IL). The enzyme-linked immunosorbent assay (ELISA) cotinine detection kit was purchased from Calbiotech (El Cajon, CA). The fibrinogen plasma detection/quantification ELISA kit was purchased from Innovative Research (Novi, MI).

**Animals**

For the WPS adult exposure C57BL/6J (10-week-old male) mice (referred to hereafter as C57BL/6) were purchased from the Jackson Laboratory (Bar Harbor, ME), and were housed in groups of 1 to 4 at 24°C, under 12/12 light/dark cycles, with access to water and food ad libitum. For the *in utero* WPS exposure 15 males and 30 female BALB/c mice (8-weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME). Female mice were randomly assigned into two groups: Clean air and WPS group. One pair of male and female mice were kept in the same cage for mating. The pregnancy was detected by vaginal plug method (Byers et al. 2012). If the female mouse was not pregnant, mating was repeated the next day. After birth, the male offspring mice were kept in clean air and monitored until the age of 10 weeks. Adult male offspring mice were divided into; (1) clean air (exposed *in utero* to clean air), (2) WPS (exposed *in utero* to WPS. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of The University of Texas at El Paso.

**Waterpipe Smoke (WPS) Whole Body Exposure Protocol**

The exposure of adult mice to waterpipe smoke (WPS) utilized a whole-body exposure chamber (40cm x 30cm x 25cm, LxWxH), which was connected to a programmable waterpipe smoking machine, as described in previous report (Khabour, Alzoubi, et al. 2012b). The smoking machine delivered 171 puffs of 530 mL volume, 2.6 s puff duration, and 17 s interpuff interval according to the Beirut Method (Katurji et al. 2010a). This method is based on the smoking behavior in real-life waterpipe/hookah settings (Hookah café), and the protocol itself has been used worldwide and in different studies (Katurji et al. 2010b, Shihadeh and Azar 2006, Khabour, Alzoubi, et al. 2012a, Khan, Sundar, and Rahman 2018, Khabour, Alzoubi, Al-Sawalha, et al. 2018, Ali et al. 2017, Nemmar, Al Hemeiri, et al. 2015, Shihadeh and Saleh 2005, Shihadeh et al. 2004, Rababa'h et al. 2016, Rababa'h et al. 2019). The clay bowl of the waterpipe used in this study was loaded with 12g of commercially available flavored tobacco (Al Fakher - Double Apple Tobacco Trading, Ajman, UAE), whose ingredients include tobacco, glycerin, molasses and natural flavor with nicotine and tar. We also conducted exposures using a second tobacco brand, namely the Starbuzz (Dale Ave Stanton, CA), and performed specific platelet function experiments (aggregation and dense granule secretion). After covering the clay bowl with Aluminum foil, quick-light charcoal briquettes (Holland Hookah Charcoal) were used to burn the tobacco. The bowl of the waterpipe was filled with about 700 mL of tap water, prior to each exposure session. Along with the smoke puff delivered to the exposure chamber by the smoking machine, fresh/clean air was continuously pumped into the chamber, resulting in an air change rate of approximately 1.5 changes per hour. Carbon monoxide (CO) concentration (average levels were 925 ppm) was continuously monitored in the chamber using an electrochemical sensor (Bacharach Monoxor Plus) drawing a flow rate of 0.3 LPM through a 25 mm glass fiber filter (Pall Type A/E). The WPS

mice were exposed, as described above, for one exposure session 60 min/day for seven days, and all experiments performed on the day of the last exposure session. As for the *in utero* WPS exposure, female mice (C57BL/6) were exposed to the same exposure protocol one week before pairing with male mice. Exposure continued during the gestation period. After birth, exposure stopped and offspring used for experiment at the age of 10 weeks. The control mice were exposed to clean air.

**Cotinine Assay**

The serum levels of cotinine, a metabolite of nicotine, were measured in both WPS and clean air exposed mice, using the Cotinine Direct ELISA kit as per the manufacturer's instructions.

**Tail Bleeding Time**

After a complete cycle of exposure, we performed tail bleeding time assay as we described before (Qasim et al. 2018, Karim, Alshbool, et al. 2015). Briefly, anesthesia was induced and WPS and clean air exposed mice were placed on a homeothermic blanket at 37°C, after which that tail was transected 5 mm from the tip using a sterile scalpel. After transection, the tail was immediately immersed in saline (37°C constant temperature), and the time to bleeding stoppage was measured. The bleeding stoppage was not considered complete until bleeding had stopped for 1 minute. If bleeding did not stop in 10 minutes, the experiment was stopped to prevent excessive blood loss from the mice, and 10 minutes was considered the cutoff bleeding time.

***In Vivo* $FeCl_3$ Carotid Artery Injury Induced Thrombosis Model**

Thrombosis studies were performed as described previously (Qasim et al. 2018, Karim, Alshbool, et al. 2015). Briefly, WPS and clean air exposed mice (8–10 weeks old) were

anesthetized with avertin (2.5%), and the left carotid artery was exposed and cleaned with normal saline (37°C) before baseline carotid artery blood flow was measured with Transonic Micro-Flowprobe (Transonic Systems Inc, Ithaca, NY). After stabilizing blood flow, 7.5% ferric chloride was applied to a filter paper disc (1-mm diameter) that was immediately placed on top of the artery for 3 minutes. Blood flow was continuously monitored for 20 minutes or until blood flow reached stable occlusion (no blood flow for 2 minutes). Data were recorded, and time to vessel occlusion was calculated as the difference in time between stable occlusion and removal of the filter paper (with ferric chloride). An occlusion time of 20 minutes was considered as the cutoff time for statistical analysis.

**Peripheral Blood Cell/Platelet Counts**

The hematology profile, including platelets and white blood cells, of the WPS/clean air exposed mice was performed on whole blood using a HEMAVET® 950FS Multi-species Hematology System from Erba® Diagnostics (Miami Lakes, FL).

**Murine Platelet Rich Plasma Preparation**

Mice were anesthetized before blood was collected from the heart, with 0.38% sodium citrate solution (Fisher Scientific, Hampton, NH) added to inhibit coagulation. Then blood was centrifuged (237g for 15 minutes) at room temperature, and the platelet-rich plasma was then collected. Platelets were counted with the HEMAVET® 950FS Multi-species Hematology System, and their count adjusted to $7\times10^7$ platelets/mL before each experiment.

**Washed Platelet Preparation**

Blood was collected form each mouse as described above and were pooled in each group followed by diluted phosphate-buffered saline, pH 7.4, in 1:1 ratio, incubated with $PGI_2$ (10

ng/mL; 5 minutes), and centrifuged at 237g for 10 minutes at room temperature. Platelet-rich plasma was recovered, and incubated with 0.37 U/mL apyrase, and 10 ng/mL $PGI_2$, platelets were pelleted at 483×g for 10 minutes at room temperature. The pellets were resuspended in HEPES/Tyrode buffer (20 mmol/L HEPES/KOH, pH 6.5, 128 mmol/L NaCl, 2.8 mmol/L KCl, 1 mmol/L $MgCl_2$, 0.4 mmol/L $NaH_2PO_4$, 12 mmol/L $NaHCO_3$, 5 mmol/L D-glucose) supplemented with 1 mmol/L EGTA, 0.37 U/mL apyrase, and 10 ng/mL $PGI_2$. Platelets were then washed and resuspended in HEPES/Tyrodes (pH 7.4). Platelets were counted using the HEMAVET® 950FS Multi-species Hematology System and adjusted to the indicated concentrations.

### *In vitro* Platelet Aggregation

The platelet-rich plasma (PRP) from WPS exposed and clean air mice were activated with thrombin (0.1 U/mL) or ADP (5 μmol/L). Platelet aggregation was measured by the turbidometric method using model 700 aggregometer (Chrono-Log Corporation, Havertown, PA). Each experiment was repeated at least 3 times with blood pooled from at least 3 different groups (i.e., at least 8 mice each) that were exposed to either WPS or clean air. The aggregation assay was conducted after exposure to the two different tobacco brands.

### Dense Granule Release

Platelet-rich plasma (250 μL; $7\times10^7$/mL) were placed into siliconized cuvettes and stirred for 5 minutes at 37°C at 1200 rpm, followed by the addition of luciferase substrate/luciferase mixture (12.5 μL, Chrono-Log), and activated the PRP using the agonists thrombin (0.1 U/mL) or ADP (5 μmol/L). The release of ATP was measured using 700 aggregometer (Chrono-Log Corporation, Havertown, PA). This assay was conducted after exposure to the two different tobacco brands.

**Flow Cytometric Analysis**

Flow cytometric analysis was carried out as described (Karim, Alshbool, et al. 2015, Qasim et al. 2018). Briefly, washed platelets ($2\times10^7$/mL) from clean air- or WPS–exposed mice were prepared as described above and stimulated with thrombin (0.1 U/mL) or ADP (5 μmol/L) for 5 minutes. Platelets were then fixed with 2% formaldehyde for 30 minutes at room temperature, followed by incubated with FITC-conjugated CD62P (P-selectin), Annexin V or phycoerythrin-conjugated rat anti-mouse JON/A antibodies at room temperature for 30 minutes in the dark. Finally, platelets were diluted 2.5-fold with HEPES/Tyrode buffer (pH 7.4). The samples were transferred to FACS-tubes and fluorescent intensities were measured using a BD Accuri C6 flow cytometer and analyzed using CFlow Plus (BD Biosciences, Franklin Lakes, NJ). Each experiment was repeated at least 3 times with blood pooled from at least 3 different groups (at least 8 mice each) that were exposed to either clean air or WPS.

**Platelet Spreading**

Platelet spreading assay was carried out as we described before (Choi, Karim, and Whiteheart 2006, Qasim et al. 2019). Briefly, sterile glass coverslips were coated with 0.2 μg/mL of fibrinogen for 30 minutes at room temperature. Washed platelets were placed onto these fibrinogen-coated coverslips before they were fixed with 3.7% (vol/vol) formaldehyde for 15 minutes and quenched with 50 mmol/L ammonium chloride. Cells were rinsed with PBS and incubated with tetramethylrhodamine-conjugated phalloidin (1 μg/mL) in 10% fetal bovine serum/PBS with 0.2% saponin. Coverslips were mounted and examined and imaged using a Leica DMi8 inverted widefield fluorescence microscope with an integrated high-precision focus drive. Images were processed using LAS X Wizard imaging software. Objective lenses used were a ×63/×100 numeric aperture. Type A immersion oil (Fryer) was used for the ×63 objective. This

experiment was repeated at least 3 times, with blood pooled from a group of 8 mice each time. Comparison is based on difference in the level of spreading.

**Immunoblotting**

Immunoblot was carried out as described before (Qasim et al. 2018, Karim et al. 2016). Briefly, clean air– or hookah/waterpipe–exposed washed platelets were stimulated with thrombin (0.1 U/mL) for 3 minutes followed by lysis with 1X sample buffer. Next, proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to Immobilon-P PVDF membranes (Bio-Rad, Hercules, CA). Membranes were then probed with the primary antibodies (ERK, pERK, Akt, and pAkt) and visualized with horseradish peroxidase–labeled antirabbit or antimouse immunoglobulin G as required. The antibody binding was detected using enhanced chemiluminescence substrate (Thermo Scientific, Rockford, IL). Images were obtained with ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA), and quantified using Image Lab Software 6.0.1 (Bio-Rad, Hercules, CA). For expression levels of PI3K (P85), PAR4, and integrin αIIbβ3, platelet proteins were separated by SDS-PAGE and transferred to Immobilon-P PVDF membranes (Bio-Rad, Hercules, CA). They were then probed with anti-PI3K, anti- PAR4, or anti-integrin αIIbβ3 primary antibodies and visualized with an appropriate alkaline phosphatase–coupled secondary antibody using enhanced chemifluorescent substrate (Thermo Scientific, Rockford, IL).

**Mouse Treatment with Nicotine and Cotinine**

Mice were also injected with 1 μM cotinine (a “real-life” dose selected based on waterpipe literature) (Kassem, Kassem, Liles, Jackson, et al. 2018, Moon et al. 2018, Nosratzehi et al. 2015, Kassem et al. 2014) or vehicle once daily for one week. Experiments on cotinine treated animals

were performed on the day of the last injection. As for nicotine, mice were injected with 1 mg/kg nicotine, as previously used in the literature (Ljungberg et al. 2013), and the blood collected after one hour post injection.

**Fibrinogen Assay**

The plasma concentration of fibrinogen was determined using ELISA kit as per the manufacturer's instructions.

**Statistical Analysis**

All experiments were performed at least 3 times with blood pooled from 3 groups of at least 8 mice each time, as applicable. Data analysis was performed using GraphPad PRISM. V7 statistical software and presented as mean ± standard deviation (SD). The Mann-Whitney test was used for the evaluation of differences in mean occlusion and bleeding times, whereas the one-way ANOVA with Tukey's multiple comparisons test as post hoc was used for the analysis of the flow cytometry data, as applicable (per number of groups). Significance was accepted at $P < 0.05$ unless stated otherwise.

**Results**

**Adult WPS Exposure Systemically Delivers Nicotine In Exposed Mice.**

As a means of validating the WPS exposure protocol, the serum levels of the nicotine metabolite cotinine (Benowitz, Hukkanen, and Jacob 2009) are typically measured in exposed animals and/or “tobacco users”. Hence, our results showed significant increases of cotinine levels in the WPS exposed mice (15.2 ± 0.72 ng/mL; Fig 2.1), in serum obtained immediately after the conclusion of the exposures, whereas it was undetectable in that from the clean air controls. These levels overlap with those found in human waterpipe users (5.5-77.8 ng/mL) (Nosratzehi et al. 2015, Moon et al. 2018, Macaron et al. 1997, Levine et al. 2013, Kassem, Kassem, Liles, Jackson, et al.

2018). Our data support the notion that our adult WPS exposure protocol results in the systemic delivery of nicotine into the exposed mice.

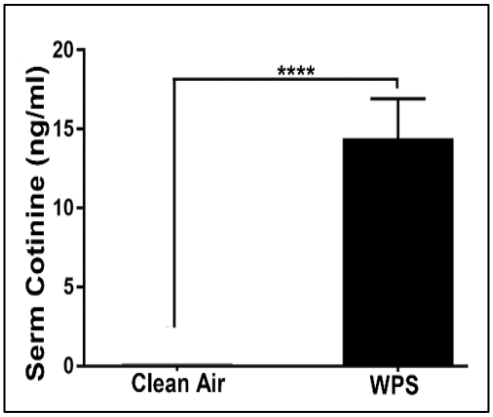



Figure 2.1**: WPS increases cotinine concentration in exposed mice**. The concentrations of cotinine were measured in serum from WPS and clean air exposed mice. Each bar represents the mean ± SD (n = 10; ****P<0.0001)

On the other hand, cotinine was not detected in the serum of the *in utero* WPS exposed offspring mice. This would be expected as those animals were exposed only *in utero* and examined at 10 weeks of age, when cotinine is expected to be completely cleared from their system.

**Adult WPS Exposure Modulates Hemostasis and Thrombosis Development.**

Numerous studies showed that cigarette smoking causes disease states that are linked to thrombosis (Ambrose and Barua 2004a, Levine 1973). On contrast, it is yet to be determined whether waterpipe smoking carries a similar effect. Therefore, we initially investigated the effects of waterpipe smoke (WPS) exposure on both hemostasis and thrombogenesis. As for its effect on hemostasis, the tail bleeding time assay revealed that time to bleeding stoppage was significantly shortened in the WPS-exposed mice when compared with those exposed to clean air, specifically 11.43 ± 2.15 seconds to 300.7 ± 7.32 seconds, respectively (Figure 2.2A). This line of evidence indicates a prothrombotic phenotype in the WPS-exposed mice. Thus, we sought next to examine if these are more prone to thrombosis. Using the ferric chloride carotid artery injury-induced

thrombosis model, we indeed found that the WPS-exposed mice had a shortened time for occlusion, in comparison with the controls, specifically, 72 ± 33.7 seconds to 370 ± 62.9 seconds, respectively (Figure 2.2B). These data document, for the first time, that adult WPS exposure directly modulates hemostasis and thrombus occlusion.

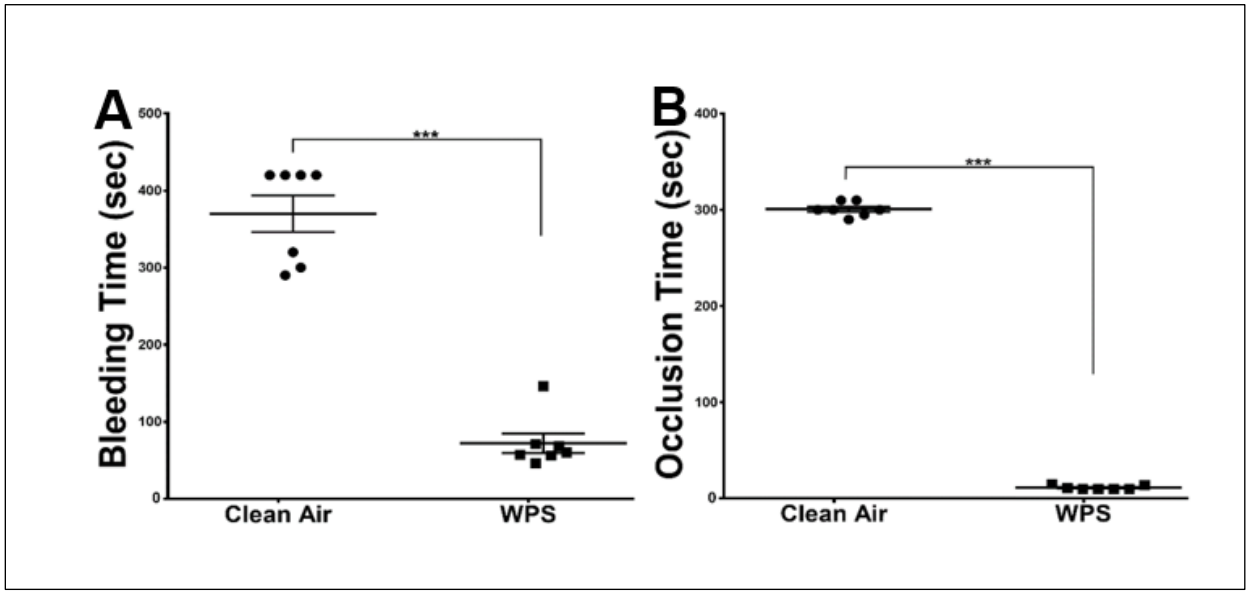



Figure 2.2**: Exposure to WPS alters hemostasis and thrombosis. (A)** This data illustrates the results of tail bleeding time assay (as described in the "Methods") comparing WPS and clean air exposed mice. Each point represents the tail bleeding time of a single animal (clean air, n=7; and WPS, n=7; ***P<0.001). **(B)** This data illustrates the results of the ferric chloride–induced thrombosis model (as described in the "Methods") comparing WPS and clean air exposed mice time to occlusion. Each point represents the occlusion time of a single animal (clean air, n=7; and WPS, n=7; ***P<0.001).

### *In utero* WPS Exposure Modulates Hemostasis and Thrombosis Development.

It is well established that prenatal/during pregnancy cigarette smoking is associated with negative health outcomes on the offspring in adult life (Law et al. 2003, Prabhu et al. 2010, Vardavas et al. 2010). Thus, we sought to investigate the effect of *in utero* WPS exposure on hemostasis and thrombogenesis. Our results illustrate that offspring exposed to WPS *in utero* exhibit shortened occlusion (Figure 2.3A) and bleeding times (Figure 2.3B) in comparison to clean air exposed mice (only male mice used). These results are consistent with those of the adult exposure and indicate that the prothrombotic phenotype persists when the exposure takes place during pregnancy.

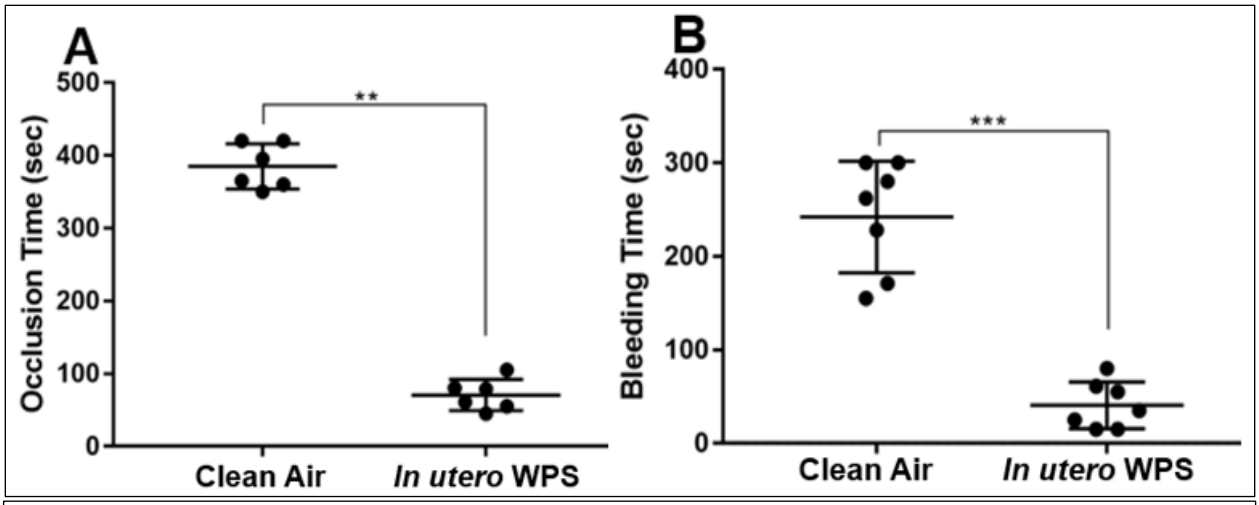



Figure 2.3**: *In utero* exposure to WPS alters hemostasis and thrombosis.** (A) This data illustrates the results of the ferric chloride–induced thrombosis model (as described in the "Methods") comparing *in utero* WPS and clean air exposed offspring mice time to occlusion. Each point represents the occlusion time of a single animal (clean air, n=6; and *in utero* WPS, n=6; **P<0.05). (B) This data illustrates the results of tail bleeding time assay (as described in the "Methods") comparing *in utero* WPS and clean air exposed mice. Each point represents the tail bleeding time of a single animal (clean air, n=7; and *in utero* WPS, n=7; ***P<0.001).

**Acute Adult WPS Exposure Does Not Affect Peripheral Blood Cell Counts.**

To exclude the possibility that changes in platelet number may have contributed to the hemostasis and thrombosis phenotype we observed, platelets were counted in the WPS and clear air mice. We did not observe any significant difference in the platelet count (Table 1) between the WPS-, and clean air-exposed mice, namely (556 ± 24.3 to 573 ± 31.2 (thousand/µL; P=0.40). These results indicate that under the present experimental conditions, the prothrombotic phenotype is independent of platelet count. Moreover, we did not observe any detectable effects for WPS on the counts of white/peripheral blood cells (Table 1).

Table 2.1: Complete blood count for adult WPS exposed mice.

| Cell type | WPS (mean ± SD) | Clean Air (mean ± SD) | P Values |
|---|---|---|---|
| Platelets (k/mL) | 556 ± 24.30 | 573 ± 31.20 | 0.40 |
| MPV (fl) | 4.8 ± 0.26 | 4.90 ± 0.10 | 0.54 |
| Red blood cells (M/mL) | 8.15 ± 0.85 | 8.05 ± 0.90 | 0.88 |
| Lymphocytes (k/mL) | 1.77 ± 0.05 | 1.83 ± 0.37 | 0.76 |
| Monocytes (k/mL) | 0.07 ± 0.01 | 0.10 ± 0.02 | 0.17 |
| Granulocytes (k/mL) | 2.29 ± 0.10 | 2.50 ± 0.30 | 0.30 |
| HCT (%) | 40.4 ± 1.45 | 39.45 ± 2.11 | 0.72 |

***In utero* WPS Exposure Does Not Affect Peripheral Blood Cell Counts.**

Our results were again consistent with the adult exposure, and we did not observe any significant difference in the platelet count (Table 2) between the *in utero* WPS, and clean air-exposed mice, namely (599 ± 60.87 to 571 ± 83.94 (thousand/µL; P=0.46). These results indicate that under the present experimental conditions, the prothrombotic phenotype seen in the *in utero* exposed offspring is independent of platelet count. Moreover, we did not observe any detectable effects for WPS on the counts of white/peripheral blood cells (Table 2).

Table 2.2: Complete blood count for the *in utero* WPS exposed mice.

| Cell type | Clean air (mean ± SD) | *In utero* WPS (mean ± SD) | P Values |
|---|---|---|---|
| Platelets (k/mL) | 571.5±83.94 | 599.3±60.87 | 0.46 |
| MPV (fl) | 5.03±0.15 | 5.125±0.1832 | 0.31 |
| Red blood cells (M/mL) | 5.52±1.19 | 5.049±0.8204 | 0.36 |
| Lymphocytes (k/mL) | 1.45±0.42 | 1.323±0.3208 | 0.46 |
| Monocytes (k/mL) | 0.06±0.01 | 0.075±0.010 | 0.29 |
| Granulocytes (k/mL) | 1.82± 0.56 | 1.736± 0.562 | 0.71 |
| HCT (%) | 26.84±6.06 | 24.79±4.04 | 0.43 |

**Adult WPS Exposure Enhances Agonist-Induced Platelet Aggregation.**

Previous studies have shown that cigarette smoking enhances platelet function (Takajo et al. 2001). Given that our data showed a robust prothrombotic phenotype in WPS-exposed mice, we hypothesize that this will derive, in part, from platelet hyperactivity as a result of WPS exposure. Thus, we sought to examine the effects of WPS exposure on agonist-induced platelet aggregation. It was found that platelets from WPS-exposed mice exhibited enhanced platelet aggregation in comparison to those from clean air controls, in response to thrombin or ADP (Figure 2.4A & 2.4B; inset shows quantification of data). Together, the hyperactive platelet state is consistent with shortened bleeding and occlusion times phenotype observed in the WPS mice. A similar result was

obtained when the same experiment was repeated with a different waterpipe tobacco brand (see Methods for details; Figure 2.4C & 2.4D; inset shows quantification of data).

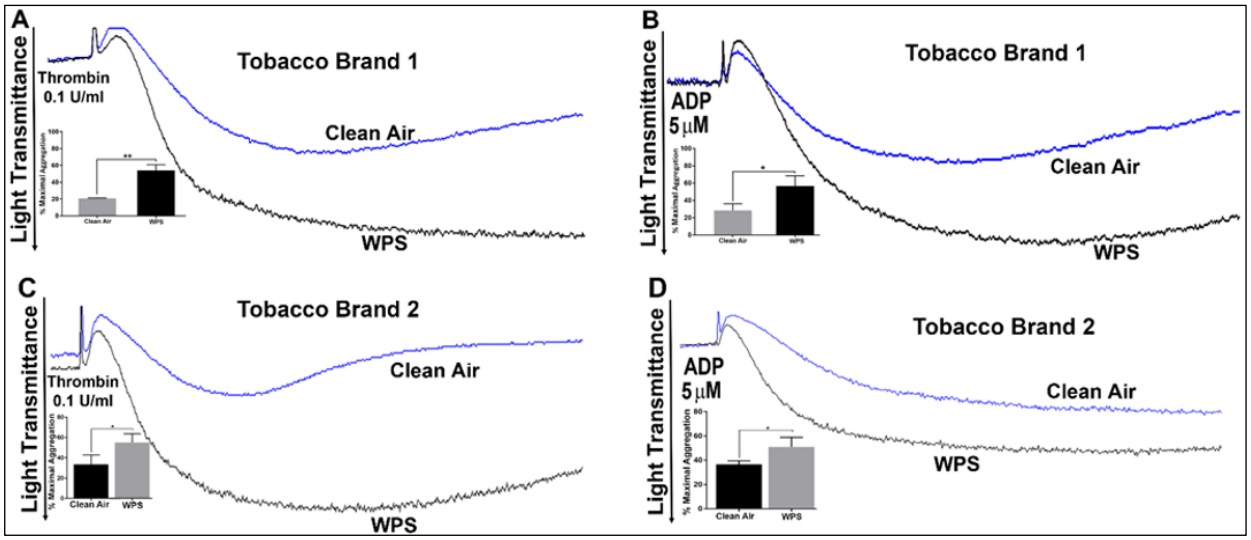



Figure 2.4**: Platelet aggregation is enhanced in WPS exposed mice, using two different brands**. Platelets from WPS (two separate brands) and clean air exposed mice were stimulated with 0.1 U/mL thrombin, or 5 μmol/L ADP, before their (A, B, C, D) aggregation responses were measured in a lumi-aggregometer. The experiment was repeated 3 times, with blood pooled from at least 8 mice each time (*P <0.05; **P <0.01).

### *In utero* WPS Exposure Enhances Agonist-Induced Platelet Aggregation.

Consistent with the results that showed *in utero* exposure to WPS produces a prothrombotic phenotype, and in agreement with the effect seen in the adult exposure on platelet aggregation, our data revealed that platelets from *in utero* WPS-exposed mice exhibited enhanced aggregation in comparison to those from clean air controls, in response to thrombin or ADP (Figure 2.5 A & B).

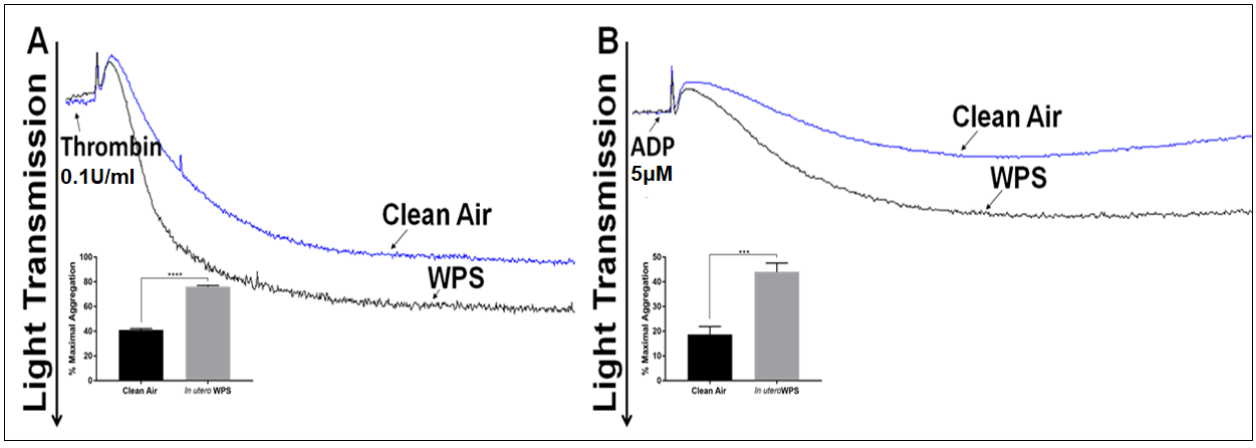


Figure 2.6**: Adult WPS exposure enhances platelet granule release**. (A, B, C, and D) Dense granule secretion response was measured in a lumi-aggregometer. Platelets were incubated with luciferase (12.5 µL) for the dense granules measurements. (*P <0.05; **P <0.01). (E, F) Alpha granule secretion was measured by incubating platelets with P-selectin (for α granules), before the fluorescent intensities were measured by flow cytometry after stimulation with 0.1 U/mL thrombin or 5 µmol/L ADP. Each experiment was repeated 3 times, with blood pooled from at least 8 mice each time.

**Adult WPS Exposure Enhances Agonist-Induced Platelet Secretion.**

We next sought to investigate the impact of WPS exposure on agonist-induced platelet secretion, given the importance of the platelet granule release in amplifying the initial platelet response (Dawood, Wilde, and Watson 2007, Storrie and Whiteheart 2017). Our results demonstrated dense granule/ATP secretion is enhanced in the WPS platelets, in response to thrombin or ADP (Figure 2.6A, 5B), even with exposure to a different tobacco brand (Figure 2.6C, 5D), which is consistent with the aggregation data. Similarly, platelets from mice exposed to WPS had significantly higher expression of P-selectin on their surface in response to 0.1 U/mL of the agonist thrombin or 5µM ADP (Figure 2.6E & 2.6F).

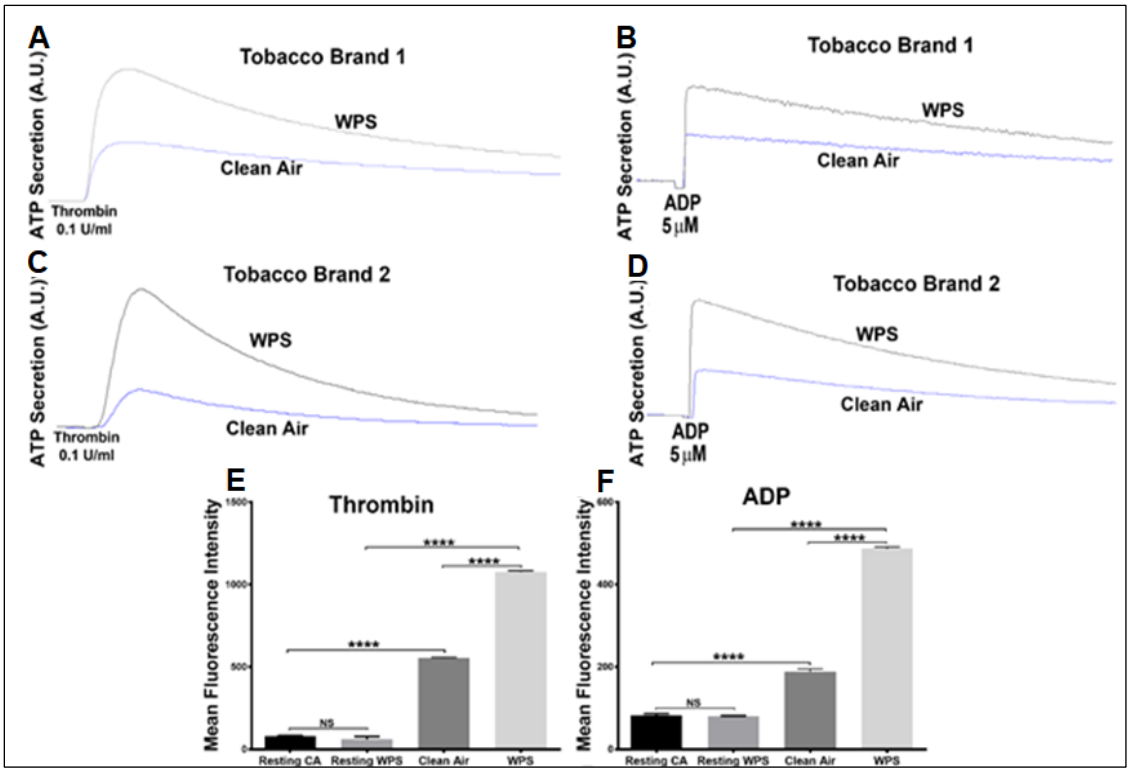


Figure 2.5**: Platelet aggregation is enhanced in *in utero* WPS exposed mice**. Platelets from *in utero* WPS and clean air exposed mice were stimulated with 0.1 U/mL thrombin, or 5 µmol/L ADP, before their (A, B) aggregation responses were measured. The experiment was repeated 3 times, with blood pooled from at least 8 mice each time.

These findings indicate that whole-body exposure to WPS enhances both dense and α-granule secretion, which is in line with the notion that these platelets are hyperactive.

### *In utero* WPS Exposure Enhances Agonist-Induced Platelet Secretion

In agreement with aggregation data, dense and alpha granules secretion were found to be enhanced in platelets from *in utero* WPS exposed mice in comparison to clean air (Figure 2.7 A, B, and C). This data suggest that platelet function is impacted by *in utero* WPS exposure, and this mechanism- at least in part- contributes to the observed prothrombotic phenotype.

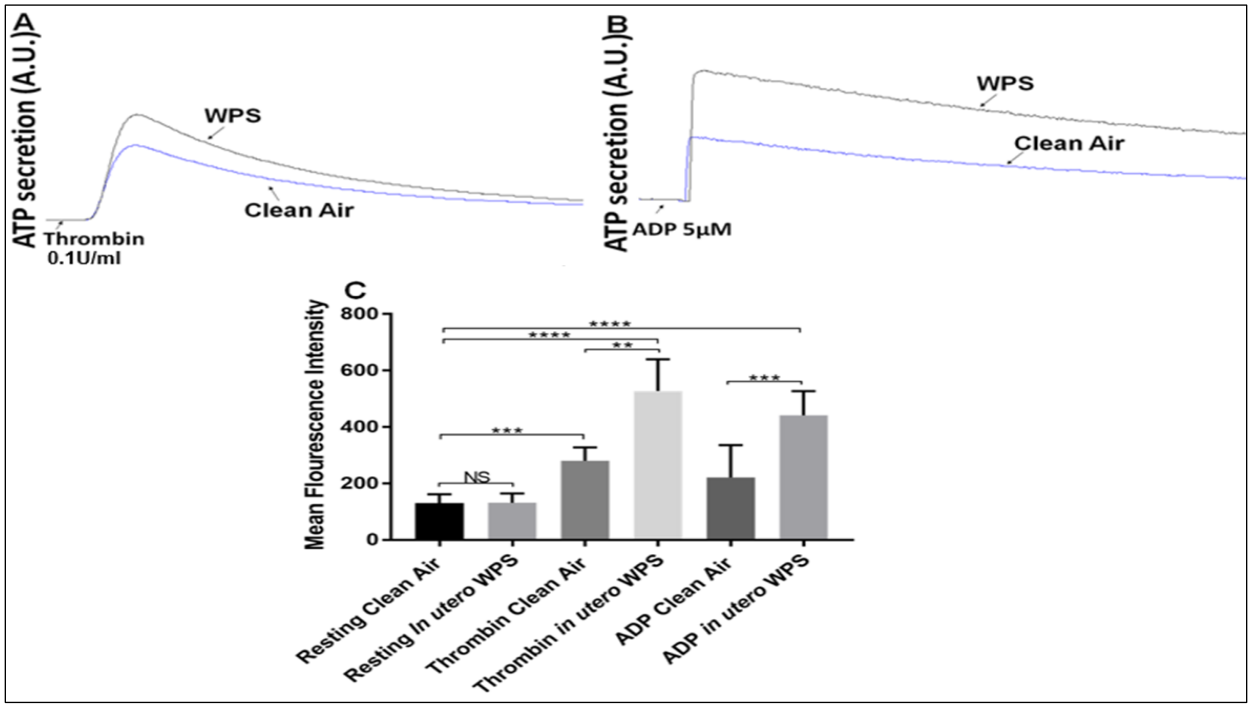



**Figure 2.7**: ***In utero* WPS exposure enhances platelet granule release.** (A, B) Dense granule secretion response was measured in a lumi-aggregometer. Platelets were incubated with luciferase (12.5 µL) for the dense granules measurements. (*P <0.05; **P <0.01). (C) Alpha granule secretion was measured by incubating platelets with P-selectin (for α granules), before the fluorescent intensities were measured by flow cytometry after stimulation with 0.1 U/mL thrombin or 5 µmol/L ADP. Each experiment was repeated 3 times, with blood pooled from at least 8 mice each time.

**Adult and *in utero* WPS Exposure Enhances Agonist-Induced Integrin αIIbβ3 Activation.**

In light of the notion that both adult and *in utero* WPS exposure is responsible for the hyperactive platelet phenotype, including enhanced aggregation, we next determined if the integrin GPIIb/IIIa (αIIbβ3) activation would also be potentiated. Indeed, our data revealed that activation of the αIIbβ3 integrin is enhanced in both adult (Figure 2.8A, 2.8B) and *in utero* WPS exposed platelets (Figure 2.8C, 2.8D), in contrast with the clean air controls, in response to 0.1 U/mL thrombin or 5 μM ADP. These results are consistent with the enhanced aggregation shown earlier.

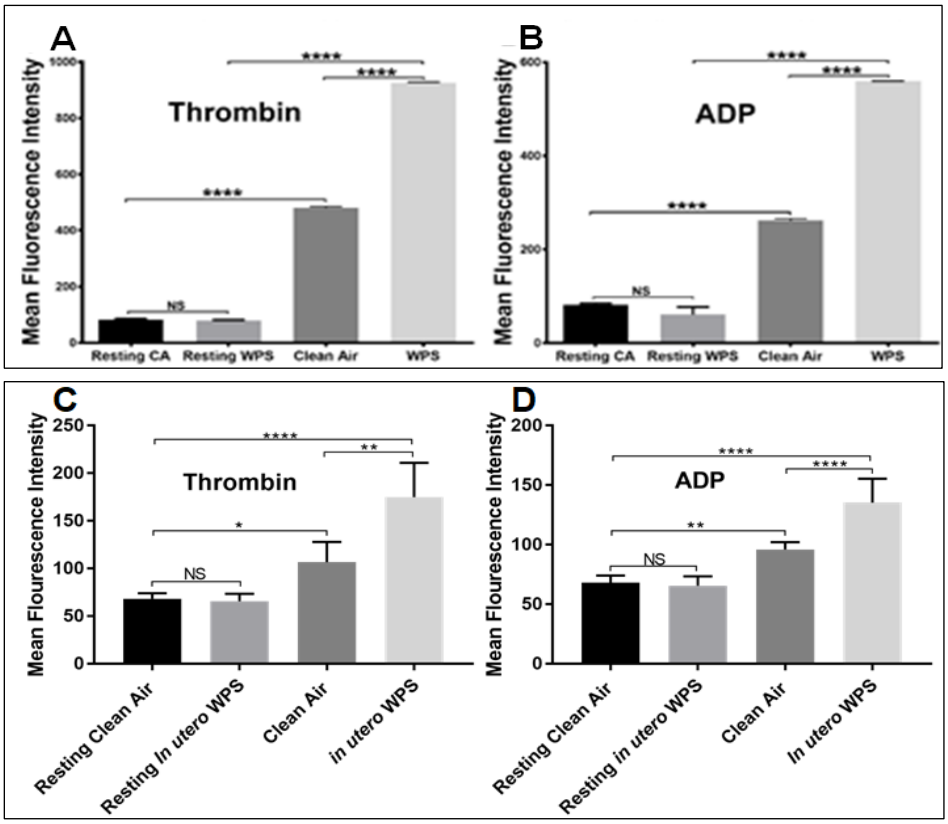



Figure 2.8**: Adult and *in utero* WPS exposure enhance platelet integrin activation.** Platelets form adult (A, B) and *in utero* (C, D) WPS or clean air exposed platelets were incubated with JON/A antibody, and the fluorescent intensities were measured by flow cytometry after stimulation with 0.1 U/mL thrombin or 5 μmol/L ADP. Average mean fluorescence intensities shown (****P < 0.0001; NS. nonsignificant). Each experiment was repeated 3 times, with blood pooled from at least 8 mice each time.

**Adult and *in utero* WPS Exposure Enhances Phosphatidylserine Expression.**

Phosphatidylserine (PS) exposure provides the essential platelet "platform" upon which the assembly of coagulation factor complexes take place (Hawiger 1987). Therefore, it is important

to assess any changes in PS expression that may result from WPS exposure. Our data showed that PS expression was significantly higher in the adult and *in utero* WPS-exposed platelets, compared to those from the clean air-exposed mice, upon stimulation with 0.1 U/mL thrombin or 5 μM ADP (Figure 2.9A, B, C, and D).

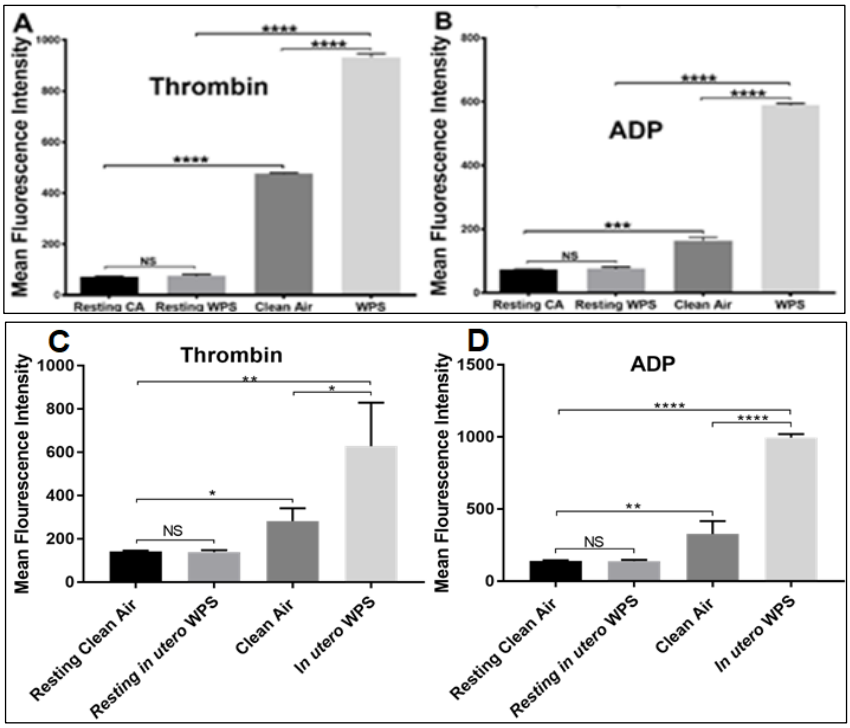



**Figure 2.9: Platelet phosphatidylserine exposure is enhanced in both adult and *in utero* WPS exposed mice**. Adult (A, B) and *in utero* (C, D). Average mean fluorescence intensities shown (****P < 0.0001; NS. nonsignificant). Each experiment was repeated 3 times, with blood pooled from at least 8 mice each time.

Taken together, our data thus far support the notion that exposure of platelets to WPS, in adulthood or *in utero*, produces a hyperactive state, in comparison to clean air. This hyperactive state (enhanced function) was in the form of enhanced aggregation, secretion, integrin activation, and phosphatidylserine exposure.

**Adult WPS Exposure Enhances Platelet Spreading.**

Platelet spreading is a vital aspect of hemostatic plug formation and thrombosis (Hawiger 1987). To this end, and upon vessel injury, platelet activation mediated via a host of receptor-induced cascades, triggers rapid reorganization of the actin cytoskeleton, thereby resulting in platelet rounding and adhesion to the surface. Consequently, platelet morphological changes occur, e.g. filopodia and lamellipodia formation, which ultimately strengthens contact with the surface as well as to other platelets. As for the impact of adult WPS exposure on spreading, filopodia and lamellipodia formation is higher/potentiated in the WPS-exposed platelets, compared to those from the clean air-exposed mice, upon stimulation with 0.1 U/mL thrombin (Figure 2.10). This finding further supports the notion that WPS results in a hyper-state of platelet activity.

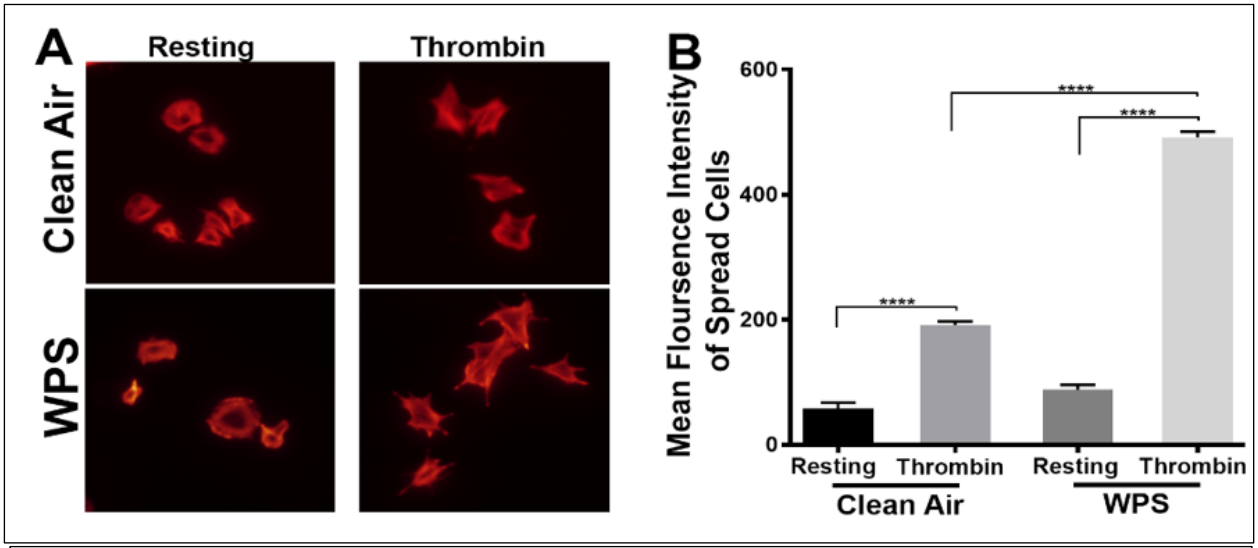
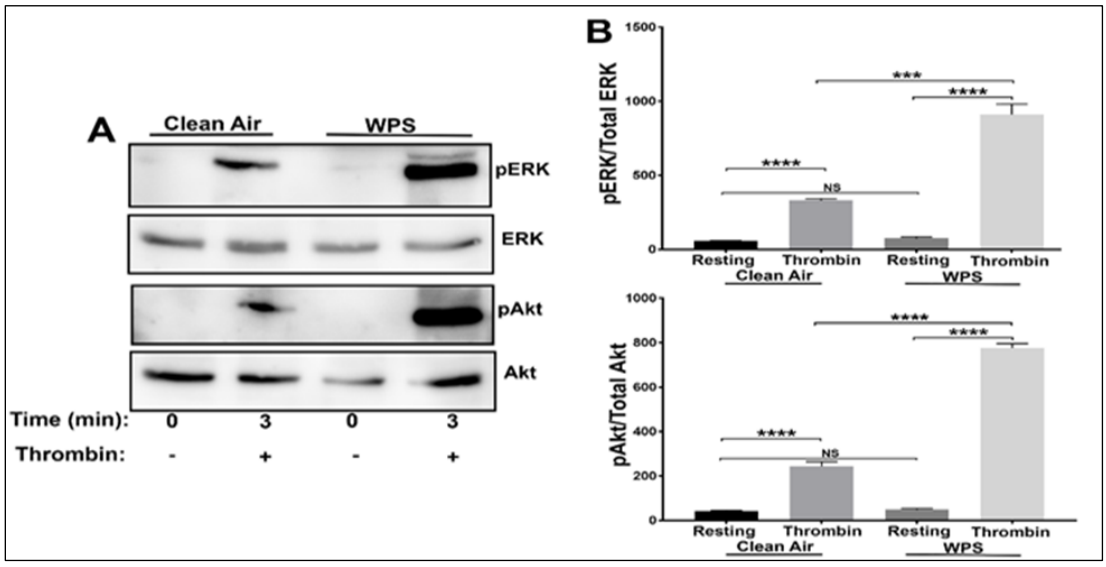



**Figure 2.10**: **Platelet spreading is enhanced in WPS exposed mice.** Platelets from adult WPS and clean air exposed mice were allowed to adhere to fibrinogen-coated coverslips for 5 minutes after stimulation with thrombin (0.1 U/mL). (A) Tetramethylrhodamine-conjugated phalloidin was used to stain for F-actin and Images were processed using LAS X Wizard imaging software. (B) Quantification of the spreading data. Data are representative of 3 independent experiments.

**Adult WPS Exposure and Nicotine Enhance Agonist-Induced Akt and ERK Phosphorylation.**

A critical signaling mechanism in platelet function and thrombus formation is the phosphorylation of Akt and/or ERK proteins (Senis et al. 2003, Stojanovic et al. 2006, Garcia et al. 2005, Woulfe et al. 2004). Therefore, we investigated whether WPS exposure would enhance phosphorylation of Akt and/or ERK proteins in platelets. Our data showed that Akt and ERK phosphorylation are enhanced in adult WPS-exposed platelets, relative to controls after stimulation with 0.1 U/mL thrombin (Figure 2.11A; data quantification is shown in Figure 2.12B). These results provide biochemical evidence and indicate that Akt and/or ERK are an essential component in the WPS-mediated modulation of platelets toward a hyperactive state. We next determined whether nicotine, the WPS chemical, has the capacity to trigger Akt and/or ERK activation. Indeed, platelets from mice that were injected with nicotine (1 mg/kg IV) exhibited enhancement of Akt and ERK phosphorylation in response to 5 μM ADP (Figure 2.11C; data quantification is shown in Figure 2.11D); which is consistent with previous reports (West et al. 2003, Brunzell, Russell, and Picciotto 2003, Dajas-Bailador, Soliakov, and Wonnacott 2002). This data suggests that nicotine plays a role "at least in part" in the priming of platelets into a hyperactive state.



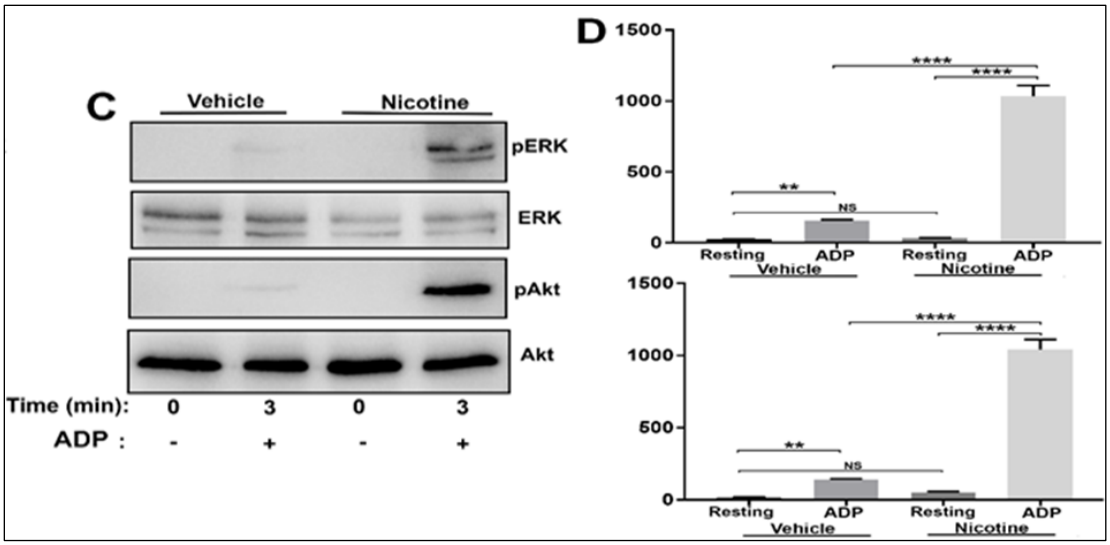



**Figure 2.11**: **Platelet Akt and ERK activation (phosphorylation) are enhanced in adult WPS exposed and nicotine treated mice**. Platelets from WPS and clean air exposed mice as well as from nicotine and vehicle injected mice were prepared and washed. Platelets were stimulated with 0.1 U/mL thrombin (A) or 5 μmol/L ADP (C) for 3 minutes, and proteins were lysed using 1X sample buffer. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis before being subjected to immunoblotting with anti-Akt, anti-pAkt (Ser473), anti-ERK, and anti-pERK antibodies. (B. D) Data quantification of agonist-induced ERK and Akt phosphorylation (****P < 0.0001; ***P < 0.001; NS. nonsignificant). Each experiment was repeated at least 3 times, with blood pooled from a group of 8 mice each time. WPS indicates Waterpipe Smoke.

**Adult WPS Exposure Does Not Modulate Plasma Fibrinogen Levels.**

To determine if the coagulation system is impacted by WPS, we sought to see if there will be any change in the plasma concentration of fibrinogen. Our results showed that the plasma concentration of fibrinogen was not affected in WPS exposed mice in comparison to clean air under the present experimental conditions (Figure 2.12).

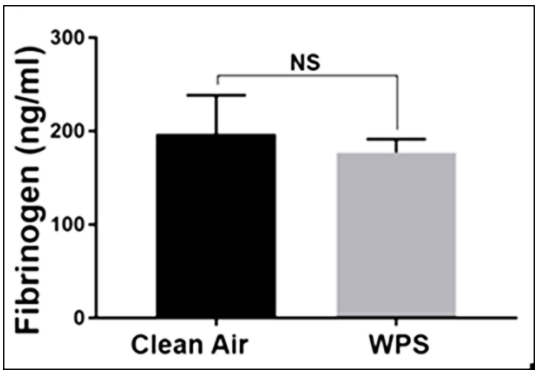



**Figure 2.12**: **WPS exposure does not affect plasma fibrinogen.** Plasma was collected from WPS and clean air controls before the levels of fibrinogen were measured, NS. nonsignificant. Each experiment was repeated at least 3 times, with blood pooled from a group of 8 mice each time

**Cotinine Enhances Platelet Aggregation and Secretion.**

Previous studies suggested that cotinine, the main metabolite of nicotine (with a long half-life) can induce thrombus formation (Sastry and Gujrati 1994). Therefore, we sought to investigate the effect of cotinine on platelet function (aggregation and secretion *ex vivo*). Our data revealed that cotinine (1 μM injection for one week) did, in fact, enhance thrombin-induced platelet aggregation and dense granule secretion, in comparison to the vehicle control (Figure 2.13A & 2.13B). These data indicate that cotinine is a likely key contributor to WPS's "negative" effects on platelets.

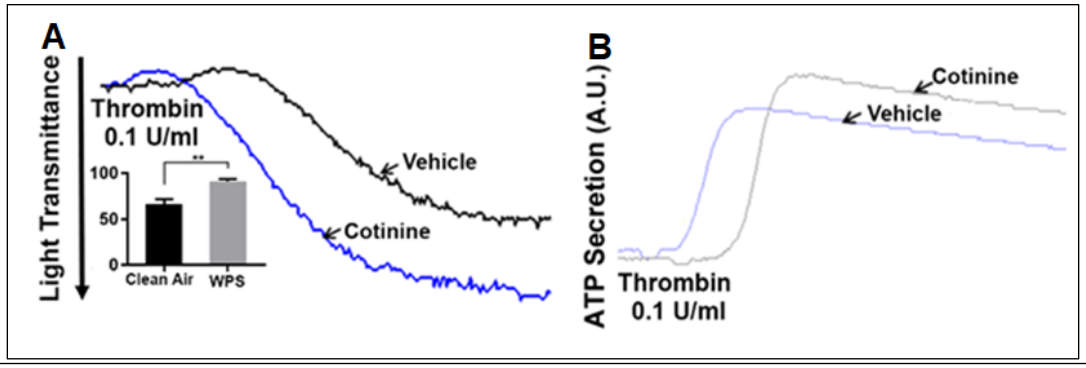



**Figure 2.13**: **Cotinine enhances platelet aggregation and dense granule secretion.** Mice were injected with 1 μM cotinine or vehicle, once daily for 1 week before platelets were harvested, and stimulated with thrombin (0.1 U/mL), and aggregation **(A)** and ATP release **(B)** were monitored using Lumi-aggregometer. The experiment was repeated 3 times, with blood pooled from at least 8 mice each time.

**Adult WPS Exposure Does Not Modulate Expression of Integrin αIIbβ3, PAR4, or PI3K.**

We next sought to investigate if the aforementioned effects of WPS may involve changes in the expression levels of certain platelet proteins, namely integrin αIIbβ3 (GPIIb/IIIa), PAR4, and PI3K/p85. To this end, we did not observe any detectable changes in the expression levels of integrin αIIbβ3, PAR4 or PI3K/p85, between WPS and clean air platelets (Figure 2.14A; data quantification is shown in Figure 2.14B). These data suggest that, at least under the current experimental conditions, short-term exposure to WPS does not alter the expression of platelet proteins.

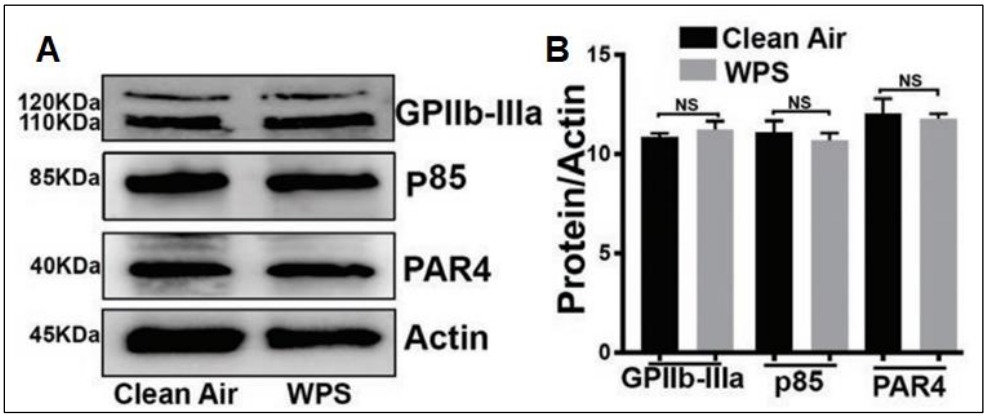



**Figure 2.14**: **WPS exposure does not modulate the expression of platelets protein.** Platelets from WPS and clean air–exposed mice were prepared and washed. Proteins were lysed using 1× sample buffer, and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis before being subjected to immunoblotting with anti-GPIIb-IIIa (αIIbβ3), anti-PAR4, or anti-p85/PI3K antibodies; (E) data quantification of GPIIb-IIIa, PAR4, and p85/PI3K expression; NS, nonsignificant. Each experiment was repeated at least 3×, with blood pooled from a group of 8 mice each time.

## Discussion & Conclusion

In this study, we investigated the effect of adult as well as *in utero* exposure to hookah/waterpipe smoke (WPS) on platelet function, hemostasis, and thrombogenesis. Similar to cigarette smoking, our results indicate that exposed mice are under increased risk for thrombosis with a shortened time for physiological hemostasis, which seems to derive from a hyperactive platelet function phenotype.

Many studies have undisputedly established that cigarette smoking is a risk factor for cardiovascular disease, including unstable angina (Daly et al. 1983, Merry et al. 2011), myocardial infarction (Critchley and Capewell 2003), and stroke (Shinton and Beevers 1989). On the other hand, despite its widespread use and being a rising trend in the west (Jukema, Bagnasco, and Jukema 2014b, Jawad et al. 2018, Salloum et al. 2017, Primack et al. 2015, Orth and Merkel 2018)- driven by its perceived "less harm"- studies on the effects of waterpipe smoking/WPS on cardiovascular disease are limited. Moreover, the direct consequences of WPS exposure in the context of platelets and thrombosis are yet to be investigated. Waterpipes carry a toxic profile that

is thought to be comparable or to even exceed that of cigarette smoking (Khabour, Alzoubi, et al. 2012b, Sepetdjian, Shihadeh, and Saliba 2008a, Sepetdjian et al. 2013, Primack et al. 2016), which would suggest that they have "similar" negative health effects. In fact, according to some studies, the smoke emitted from a single waterpipe tobacco smoking episode contains eight times the CO, three times the nitric oxides, an average of 11.5 times the acrolein, an average of 18.2 times the formaldehyde, and up to 245 times the Polycyclic aromatic hydrocarbons (PAHs)(Shihadeh 2003b, Shihadeh and Saleh 2005, Al Rashidi, Shihadeh, and Saliba 2008, Primack et al. 2016), relative to a single regular tobacco cigarette.

Based on the aforementioned considerations, and limitation of previous studies, we sought to "uncover" the effects of direct short term WPS exposure on platelet function in adult mice, and the effect of WPS exposure on platelet function of offspring mice exposed *in utero*/during pregnancy by employing a "novel" whole-body exposure protocol that simulates real-life exposure scenarios (Shihadeh et al. 2004). An important aspect of our system is the fact that it involves minimal animal handling, with the animals unrestrained and less stressed. Thus, the animal model we used in our study (mice) should provide translational results that are clinically applicable to humans, especially in the context of tobacco exposure (Morissette et al. 2014, Churg and Wright 2007), especially since the number of puffs, puff interval, etc., are based on human exposure data. Of note, this model gives us flexibility in mimicking many aspects of human exposure that cannot be directly obtained from human, due to ethical considerations and other challenges (Moolchan and Mermelstein 2002).

Therefore, the primary aim of this project was to determine the effects of 1) short-term WPS exposure on adult platelet function, hemostasis and thrombosis; and that of 2) *in utero* WPS exposure on platelet activation, and the genesis of thrombosis. Our findings revealed- for the first

time- that short term exposure to WPS in adult mice and *in utero* significantly reduced the bleeding time in exposed mice, and increased their tendency to carotid artery thrombosis. These findings provide evidence that WPS is not safe as the common misperception implies, even with a short-term exposure of only seven/7 days, and that its harmful effects are transmitted to the offspring when exposure takes place *in utero*. In addition, our data are consistent with other reports that short-term use of waterpipes disrupts normal cardiovascular function (Hakim et al. , Shaikh et al. 2008, Kadhum et al. 2014, Azar et al. 2016, Al-Kubati et al. 2006, Eissenberg and Shihadeh 2009, Hawari et al. 2013, Layoun et al. 2014), and that exposure to other forms of tobacco impacts hemostasis and thrombosis (Karim, Alshbool, et al. 2015, Sithu et al. 2010a). Notably, patients who have a history of cardiovascular disease (e.g. atherosclerotic disease) are probably at a higher risk of having negative outcomes from waterpipe use, and this is might be because the atherosclerotic vessels are more prone to thrombotic events than normal vessels.

We next investigated the mechanism by which enhanced hemostasis and thrombosis may have manifested in the adult WPS as well as *in utero* exposed mice. Importantly, since it has been shown that an increase in platelet activity plays a major role in the pathogenesis of acute myocardial infarction (MI)(Tousoulis et al. 2010) and acute stroke (Mulley et al. 1983, Cevik et al. 2013, McCabe et al. 2004) and since thrombosis is the main mechanism of smoking-related cardiovascular mortality (Barua and Ambrose 2013), this notion was investigated herein. Our results demonstrated that agonist-induced platelet aggregation, secretion (dense and α granules), integrin activation, phosphatidylserine expression, are all enhanced in both adult as well as *in utero* WPS exposed platelets. Furthermore, platelet spreading was enhanced as a result of adult WPS exposure. Moreover, a similar enhancement of platelet aggregation and dense granule secretion was observed when adult mice were exposed to a different waterpipe/hookah tobacco brand (see

Methods for details). It is noteworthy that one study in humans reported a significant increase in $TXB_2$ levels - a metabolite of the biologically active $TXA_2$- after a single waterpipe smoking session (Wolfram et al. 2003). This increase in $TXB_2$ levels would suggest an increase in platelet activity (Catella et al. 1986), which is consistent with our findings. Together, these findings support our initial hypothesis that WPS exposure (similar to cigarette smoking) modulates platelets to a state of hyperactivity, which eventually leads to the prothrombotic phenotype observed in the short-term WPS exposed adult as well as *in utero* WPS-exposed mice. Notably, there were no changes in platelet count under our adult exposure protocol. This indicates that direct short-term (7 days) exposure to WPS does not result in any change in platelet formation. This data is consistent with a report that showed an insignificant difference in platelet count in mice exposed to waterpipe smoke (Nemmar et al. 2013). Furthermore, similar to the adult studies, our results did not show any change in the platelet count in the *in utero* exposed offspring even though the *in utero* exposure lasted throughout the 21 days of gestation. However, whether platelet number would change if adult waterpipe exposure is repeated over longer periods of time (months), as was reported previously with waterpipe exposure (Nemmar, Al-Salam, Yuvaraju, et al. 2017), or is extended beyond the gestation period remains to be determined.

Given the "functional" evidence we have obtained thus far, we next examined whether platelet activation-mediated downstream signaling, namely Akt and ERK activation, would also be impacted by WPS exposure in adult mice. Our data showed an increase in the levels of phosphorylated Akt and ERK in the WPS exposed platelets in comparison to the clean air controls. Interestingly, we previously observed similar results with exposure to e-cigarette (Qasim et al. 2018). This might suggest an overlap between waterpipes and e-cigarettes with regard to the mechanism by which they enhance platelet activation, which is presumably through nicotine. The

latter notion is consistent with previous findings that nicotine upregulates Akt and ERK pathways (West et al. 2003, Dajas-Bailador, Soliakov, and Wonnacott 2002, Brunzell, Russell, and Picciotto 2003), a notion we were able to verify under our experimental conditions, i.e., platelets from nicotine treated mice showed an increase in phosphorylation of Akt and ERK. Given the short half-life of nicotine (6-7 minutes), one cannot exclude the fact that one of its metabolites could be responsible for the enhanced Akt and ERK activation.

As far as what toxic ingredient(s) in waterpipe smoke that might be responsible for the hyperactive state of platelets, several toxicants have been found including nicotine/cotinine (Shihadeh and Saleh 2005, Schubert et al. 2011, Shihadeh 2003b, Jacob et al. 2011, Shihadeh et al. 2012), aldehydes (Shihadeh et al. 2012, Al Rashidi, Shihadeh, and Saliba 2008), particulate matter (Monn et al. 2007) as well as furanic and phenolic compounds (Sepetdjian et al. 2013, Schubert et al. 2012). To this end, we found that treatment with cotinine- one of nicotine's main metabolites- is capable of priming platelets into a hyperactive state. These data support the notion that cotinine is a key toxic waterpipe ingredient, and is a likely contributor to their prothrombotic phenotype, as well as to their harmful effects on the cardiovascular system. It is noteworthy that the amounts of these toxicants vary between waterpipe compared with cigarette smoke (per cigarette/ and per pack/day) due to different heating process and charcoal combustion (Chaouachi 2009, Shihadeh et al. 2014, Calafat et al. 2004, Moldoveanu, Coleman, and Wilkins 2008). Although the effect of each one of these chemicals on platelets is not fully studied, particulate matter (Mutlu et al. 2007, Wilson et al. 2010) and the aldehyde acrolein (Sithu et al. 2010a) were indeed found to enhance platelet function. Of note, waterpipes may, in fact, result in higher levels of nicotine given the longer duration of a session, and which involves 100-200 times the volume of smoke from a single cigarette. This also applies to other toxins in tobacco (Cobb et al. 2010).

Regarding carbon monoxide (CO), which is one of the main toxic ingredients of WPS, there is a debate regarding its impact on hemostasis and platelet function. On the one hand, studies reported an inhibitory effect on hemostasis and platelet function, whereas on the other hand, other studies reported an enhancing effect on hemostasis and platelet function (Nielsen and Pretorius 2014). This controversy may be related to the variability in the exposure protocols, as well as the methods used. Nonetheless, CO may have contributed to the negative health effects we observed with WPS.

We also examined whether the adult WPS phenotype observed may involve modulation of coagulation, or changes in the basal expression of receptors and/or signaling molecules/proteins critically involved in platelet activation. Our data showed no detectable differences in the plasma levels of the clotting factor fibrinogen between the WPS and control mice. In terms of changes in the basal expression levels of proteins, our data revealed that there was no difference in the levels of the integrin αIIbβ3, PAR4 and p85 (PI3K) proteins between the control and the WPS platelets. However, one cannot exclude the possibility that other aspects of coagulation and/or that the expression of other proteins may have changed. It is also possible that different real-life exposure scenarios may lead to changes in the plasma levels of fibrinogen and/or the expression levels of platelet proteins. Of note, higher fibrinogen plasma levels were reported in long term waterpipe smokers (Seyyed Hashem Sezavar 2004), and it was previously shown that the levels of the P2Y12 receptor protein do change in megakaryoblasts and other cells (Shanker et al. 2006), in response to nicotine.

Notably, it is well documented that regular cigarette smoking and (more recently) e-cigarettes enhance platelet function and increase the risk of thrombosis (Davis et al. 1985, Qasim et al. 2018), and our present studies show “similar” negative effects from WPS. However, comparative studies regarding differences in the magnitude or potency of each form of tobacco are

lacking but clearly warranted to better understand and appreciate the relative risk associated with each form.

The present study did not address the role of sex in the negative health consequences of waterpipe, as it employed exclusively male mice to minimize the variability that would be caused by hormonal fluctuations associated with the female's reproductive cycle (Fields 2014). Moreover, it is highly likely that "non-platelet" factors are contributing to the prothrombotic phenotype associated with WPS/hookah. These issues will be the subject of future investigations.

In summary, by employing a validated animal exposure model, we are the first to investigate the impact of whole-body WPS/waterpipe exposure on platelet function, homeostasis, and thrombosis. Furthermore, we are first to report that *in utero* WPS exposure can impact hemostasis and increase the risk of thrombosis in adult offspring. In conclusion, our findings provide evidence to argue against the common/currently held beliefs regarding the perceived safety of waterpipe smoking, which is expected to raise awareness regarding the negative health consequences of this increasingly popular form of tobacco.

# Chapter III: The Impact of Inhibition of Gβγ Subunits on Hemostasis and Thrombogenesis

## Introduction

Platelets are anucleated cells responsible for maintaining hemostasis in the cardiovascular system through the formation of primary hemostatic plugs (Vilahur et al. 2018), which is a highly regulated process. In contrast, unbalanced platelet activation contributes to the initiation of thrombus formation in numerous ischemic medical conditions, such as unstable angina, myocardial infarction, and stroke (Furie and Furie 2008). Thus, there is a thin line between the state of physiological hemostasis and pathological thrombosis, with this "tiny" difference maintained by a network of diverse membrane receptors that operate under strict conditions.

Platelet membrane receptors are the point of contact between platelets and their external environment through a wide range of agonists including thrombin, thromboxane $A_2$, and ADP (Amelirad et al. 2019, Kunapuli and Daniel 1998). Platelet receptors play a direct role in platelet function, either through platelet activation, adhesion with damaged endothelial cell walls, or with other platelets to form a thrombus (Amelirad et al. 2019). G-protein coupled receptors (GPCRs) are among the main receptors that have a critical role for platelet function (Offermanns 2006). Activation of GPCRs by it agonists (thrombin, thromboxane $A_2$, and ADP), causes the replacement of the GDP by GTP on α subunit of the heterotrimeric Gαβγ complex (Gilman 1987), facilitating conformational changes of α subunit and exposing sites on both Gα and Gβγ for interactions with downstream effectors (Smrcka 2008). Much is known about Gα and its critical role on platelet function (Jin, Daniel, and Kunapuli 1998, Daniel et al. 1998, Hollopeter et al. 2001, Foster et al. 2001, Djellas et al. 1999, Offermanns 2001). On the contrary, less is known about the Gβγ subunits in platelets and their contributions to platelet function.

In humans, 5 Gβ and 12Gγ subunits have been described (Hurowitz et al. 2000), a variety that in theory can make up to 60 combinations of Gβγ dimer. However, in reality, not all these combinations exist, expressed, or can dimerize (Yim et al. 2019). Early investigations showed that Gβγ subunits function was only restricted to the negative regulation of α subunit of the heterotrimeric Gαβγ complex (Lau et al. 2013). However, further studies revealed that Gβγ regulate important effectors such as adenylate cyclase (AC), G protein-coupled receptor kinase 2 (GRK2)(Pitcher et al. 1992), phospholipase Cβ1, β2 and β3 isoforms (Camps et al. 1992, Park et al. 1993, Smrcka and Sternweis 1993), inward rectifying potassium channels (GIRK)(Logothetis et al. 1987, Nakajima, Nakajima, and Kozasa 1996), N-type calcium channels (Ikeda 1996), and most importantly phosphoinositide 3 kinases (PI3K) (Stephens et al. 1994, Stephens et al. 1997),(Guillermet-Guibert et al. 2008). It is noteworthy that in platelets, PI3K is an important effector downstream of P2Y12 and regulates αIIbβ3 activity, which is essential for thrombus formation (Cosemans et al. 2006). To this end, the Gβγ subunits have been suggested as an effector for PI3K in platelets downstream of ADP/P2Y12, mostly through a "guilt by association" approach (Kim and Kunapuli 2011), but their role was not investigated first hand.

A number of studies suggested that pharmacological targeting of Gβγ subunits can serve as a modulator of some disease-related pathological conditions. For instance, inhibiting Gβγ subunits slowed the development of inflammation (Hirsch et al. 2000, Li et al. 2000), prevented the development of cardiomyopathy and heart failure in mice (Koch et al. 1995, Rockman et al. 1998), negatively modulate opioid responses (Xie et al. 1999), attenuated addiction (Yao et al. 2003), and inhibited tumor metastasis (Bookout et al. 2003). In platelets, while inhibiting GPCRs is considered one of the most common therapeutic approaches to prevent thrombosis (Gurbel,

Kuliopulos, and Tantry 2015), whether similar therapeutic value can be achieved by inhibiting Gβγ subunits in platelets is the concern of this study.

Therefore, we investigated the role of Gβγ subunits in platelet function, hemostasis, and thrombosis using small molecule inhibitor, namely gallein. We found that inhibiting Gβγ subunits in platelets *in vitro* results in reduced platelet aggregation, secretion, integrin activation, as well as clot retraction and platelet spreading. Further, this inhibition in platelet function was translated *in vivo* to a prolonged tail bleeding time and increased occlusion time in a $FeCl_3$-induced injury model of thrombus formation. Together, our findings support the hypothesis that inhibiting Gβγ subunits modulates platelet function, hemostasis, and thrombogenesis.

## Methods

### Reagents and Materials

Gallein and DMSO were from Tocris Bioscience (Minneapolis, MN). ADP and collagen were from Chronolog Corporation (Havertown, PA), whereas $PGI_2$ was from Cayman Chemical (Ann Arbor, MI). Fibrinogen and apyrase were from Sigma Aldrich (St. Louis, MO). Microscope slides were from Fisher Scientific (Hanover Park, IL). Coverslips were from Neuvitro Corporation (Vancouver, WA). CD62P, Annexin V, and PAC-1 FITC antibodies were from BD Biosciences (San Jose, CA). All other reagents were of analytical grade.

### Animals

C57BL/6 J mice were from Jackson Laboratories (Bar Harbor, ME). All mice used for experiments were 8–10 weeks of age, and housed in groups of 1–4 at 24 °C, under 12/12 light/dark cycles, with access to water and food ad libitum. All experiments involving animals were performed in compliance with the relevant laws and institutional guidelines and approved by the Institutional Animal Care and Use Committee of The University of Texas at El Paso.

**Human Subjects**

The Institutional Review Board approved human blood studies, and donors were asked to sign a written consent.

**Platelet-Rich Plasma Preparation**

We prepared platelets as previously described (Karim et al. 2016). Blood was collected from human volunteers through a venous puncture. Blood was also collected from mice through heart puncture. Coagulation was inhibited by 0.38% sodium citrate solution (Fisher Scientific). Blood was centrifuged (237g for 15 minutes) at room temperature (RT), and the platelet-rich plasma was then collected. Platelets were counted with the HEMAVET 950FS Multispecies Hematology System, and the counts were adjusted to $7\times10^7$ platelets per mL before each experiment.

**Washed Platelet Preparation**

Blood was collected as discussed above. Platelet-rich plasma (PRP) was recovered and platelets were pelleted at 483g for 10 minutes at RT. The pellets were resuspended in HEPES/Tyrode buffer (20 mmol/L HEPES/potassium hydroxide, pH 6.5, 128 mmol/L NaCl, 2.8 mmol/L KCl, 1 mmol/L $MgCl_2$, 0.4 mmol/L $NaH2PO_4$, 12 mmol/L $NaHCO_3$, 5 mmol/L d-glucose) supplemented with 1 mmol/L EGTA, 0.37 U/mL apyrase and 10 ng/mL $PGI_2$. Platelets were then washed and resuspended in HEPES/Tyrode (pH 7.4) without EGTA, apyrase, or $PGI_2$. Platelets were counted using the HEMAVET 950FS Multispecies Hematology System and adjusted to the indicated concentrations.

***In vitro* Platelet Aggregation**

PRP from human blood samples was incubated with vehicle (DMSO) or 25-100 μM gallein for 5 min. The samples were then stimulated with 1 μmol/L ADP, or 5 μg/mL

collagen. Platelet aggregation was measured using the turbidometric method, with a model 700 aggregometry system (Chronolog Corporation, Havertown, PA).

**Flow Cytometric Analysis**

Flow cytometric analysis for P-selectin, Annexin-V or PAC1 was performed as we described before (Lin et al. 2014). Shortly, human washed platelets were incubated either with vehicle or gallein (50 μM) for 5 min and then stimulated with ADP 10 μmol/L or collagen 5 μg/mL for 3 min. Platelets were then incubated with FITC-conjugated anti–P-selectin to measure α-granule secretion, FITC-conjugated anti-Annexin V to measure phosphatidylserine exposure, or PAC-1 antibodies to measure GPIIb-IIIa activation. Finally, fluorescent intensities were measured using a BD Accuri C6 flow cytometer and analyzed using compatible software CFlow Plus (BD Biosciences, Franklin Lakes, NJ).

**Fibrin Clot Retraction Assay**

Clot retraction assay was performed as previously described (Vemana et al. 2015). Briefly, whole blood was collected and washed platelets were isolated as discussed above. $CaCl_2$ was added extemporaneously, at a final concentration of 1 mmol/L. The glass tubes that were used for aggregation (Chrono-Log Corporation) were employed for retraction assays. The washed platelets were resuspended at $1\times10^8$/mL in HEPES-Tyrode buffer (pH 7.4). Fibrinogen (500 μg/mL) was added in 0.5-mL platelets aliquots, and clot retraction was initiated by quickly adding thrombin (0.1 U/mL). The reaction was transferred to the glass tube and the reaction was set at RT. Pictures were taken at time intervals of 5 minutes up to 35 minutes using a digital camera. Comparison is based on the difference in clot size

**Spreading Assay**

Immunofluorescence microscopy was used to assess platelet spreading, as previously described (Choi, Karim, and Whiteheart 2006). In brief, washed platelets were allowed to incubate in the presence or absence of gallein (50 μM) for 5 min. Then, they were left as is (resting) or treated with 10 μmol/L ADP for 5 min at 37 °C and placed onto fibrinogen-coated coverslips for 30 min. Platelets were fixed, rinsed/washed and incubated with either FITC/TRITC-conjugated phalloidin. The coverslips were examined and imaged using a Leica DMi8 inverted widefield fluorescence microscope with integrated high-precision focus drive. Images were processed using LAS X Wizard imaging software. Objective lenses used were a ×63/×100 numeric aperture. Type A immersion oil (Fryer) was used for the ×63 objective. Comparison is based on the difference in level of spreading.

**Tail Bleeding Time**

Mice were IV injected with either gallein (20 mg/kg/twice per day) or vehicle and the tail bleeding assay was performed after 1 h, as described before (Lin et al. 2014). Briefly, mice were anesthetized before the tail was transected at a distance of 5 mm from the tip. The tail was then immediately immersed in saline maintained at 37 °C at a constant temperature, and the time to bleeding cessation was measured (observed visually).

***In Vivo* Thrombosis**

Mice were IV injected with either gallein (20 mg/kg/twice per day) or DMSO and the $FeCl_3$-induced thrombosis model was performed after 1 hour, as described before (Lin et al. 2014). An hour post injection, animals were anesthetized, and a midline incision of the skin was made and a segment of the left common carotid artery was exposed and cleaned. Baseline carotid blood flow was measured and recorded before thrombosis was caused by placing a filter paper

(0.5 × 1 mm) saturated with 7.5% ferric chloride, onto the carotid artery. The carotid blood flow was continuously monitored for 45 min after ferric chloride application, and the data was registered by a computerized data acquisition program (LabChart6, ADInstruments, Colorado Springs, CO). Time to occlusion was calculated as the difference in time between the removal of the filter paper and stable occlusion.

**Statistical Analysis**

All experiments were performed at least three times with blood from three different human donors or in case of mouse platelets, pooled from three groups of eight-ten mice. Analysis of the data was performed using GraphPad PRISM statistical software (San Diego, CA) and presented as mean ± SEM. The Mann-Whitney test was used for the evaluation of differences in mean occlusion and bleeding times, whereas the t-test was used for FACS data analysis. Significance was accepted at $P < 0.05$ unless stated otherwise.

**Results**

***In Vitro* Inhibition of Gβγ Subunits Reduces Platelet Aggregation**

To understand the role of Gβγ in platelet function, we sought to examine the impact of inhibiting Gβγ subunits on platelet aggregation. Therefore, we employed light transmittance aggregometry, and human platelets were incubated with either gallein (25 μM) or DMSO (used as a vehicle "control") for 5 min before activation with ADP. Our initial observation revealed that platelets incubated with gallein showed reduced ADP-induced platelet aggregation when compared to vehicle (Figure 3.1A). This inhibition was found to be dose-dependent as 50 μM of gallein produced further inhibition of ADP induced platelet aggregation. Surprisingly, collagen-induced aggregation was also inhibited in platelets incubated with gallein (50 μM), when compared with vehicle (Figure 3.1B). Collagen/GPVI-mediated platelet activation (non-GPCR) is thought to

involve feedback pathways such as ADP, which in turn can act in an autocrine/ paracrine manner on their cognate GPCRs to amplify aggregation and secretion.

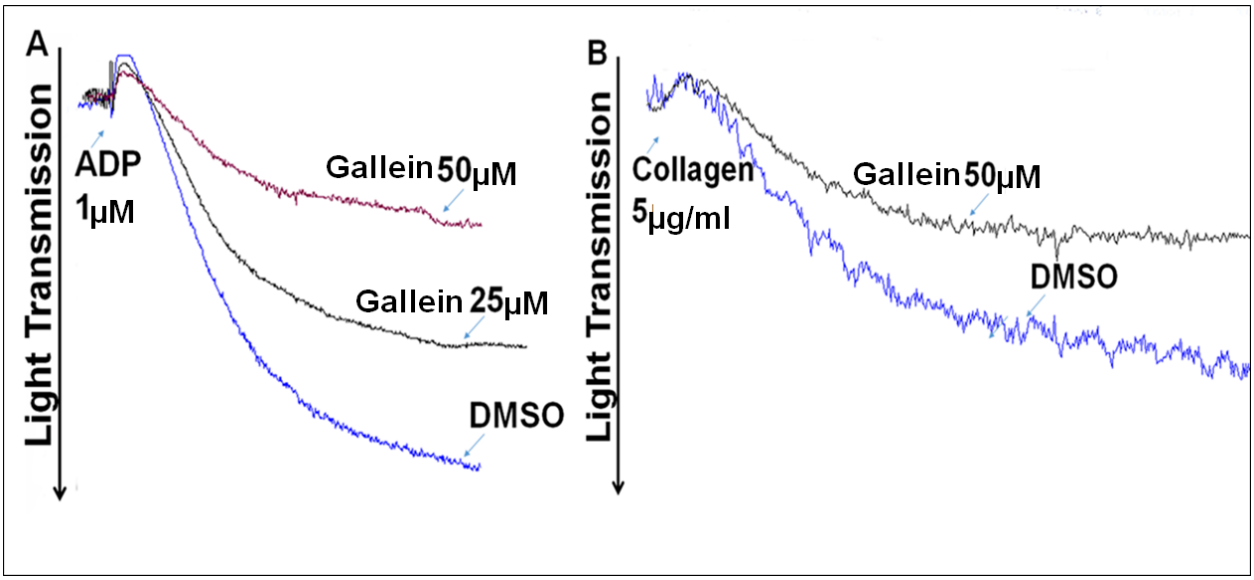



**Figure 3.1**: **Platelet aggregation is inhibited by gallein**. Platelets from gallein and vehicle treated mice were stimulated with 1 μmol/L ADP (A), or 5 μg/ml collagen (B), before their aggregation responses were measured. The experiment was repeated 3 times, with blood pooled from at least 8 mice each time.

**Inhibition of Gβγ Subunits Reduces Platelet Secretion.**

Platelet secretion plays an important role in amplifying platelets' initial response. Thus, we sought to investigate the impact of Gβγ on platelet secretion. As was the case with aggregation, ADP-induced dense granule secretion was inhibited in human platelets incubated with gallein (25 μM) in comparison to vehicle. This inhibition was dose-dependent as 50 μM of gallein further lowered the secretion induced by ADP (Figure 3.2A). Similarly, we observed inhibition in α-granule secretion (P-selectin; Figure 3.2C). We also found that collagen-induced platelet dense granule (Figure 3.2B), as well as α-granule secretion (Figure 3.2D), is inhibited when human platelets are incubated with gallein (50 μM) in comparison to vehicle.

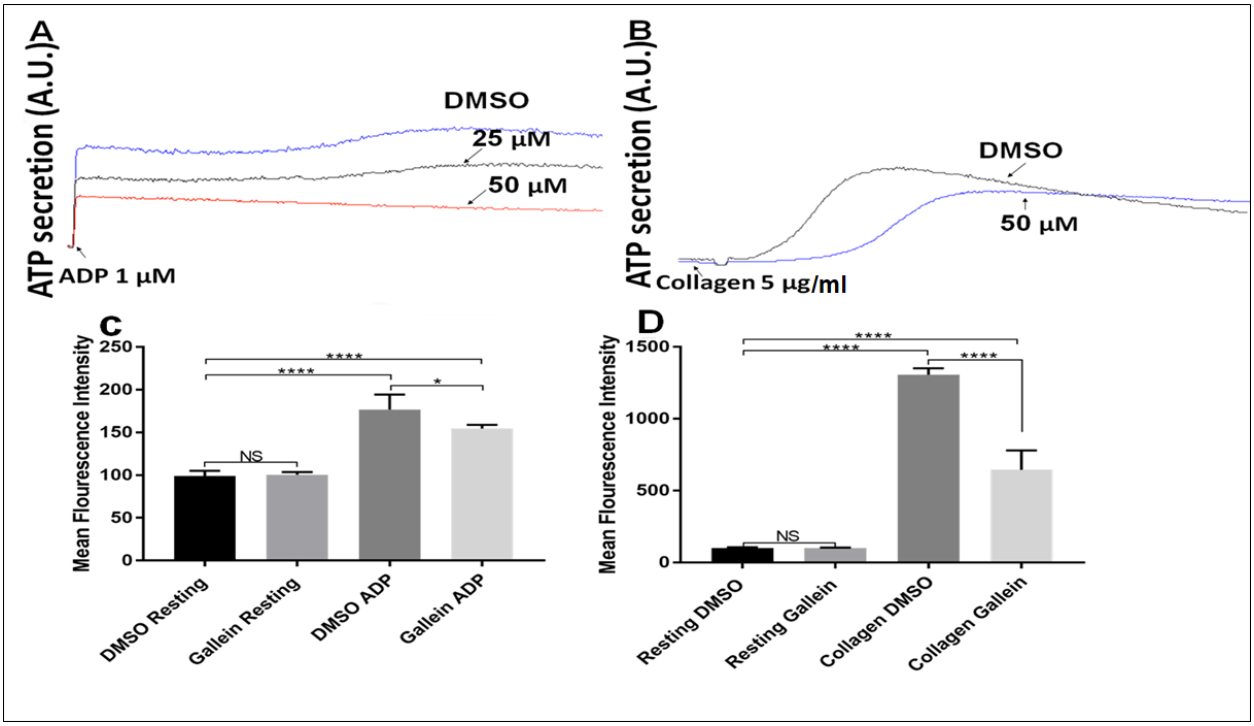



**Figure 3.2**: **Gallein inhibits platelet dense and alpha granules release**. (A, B) Dense granule secretion responses were measured in a lumi-aggregometer. Platelets were incubated with luciferase (12.5 µL) for the dense granules measurements. (C, D) Alpha granule secretion was measured by incubating platelets with P-selectin (for α granules), and the fluorescent intensities were measured by flow cytometry after stimulation with 5 µmol/L ADP or 5µg/mL collagen. The experiment was repeated 3 times, with blood pooled from at least 8 mice each time.

Overall, these results indicate that inhibiting Gβγ subunits has the capacity to suppress ADP- induced platelet aggregation/secretion. These results support our initial hypothesis that Gβγ subunits play an important role in platelet activity given the fact that Gβγ subunits convey signals downstream GPCRs; and the latter are known to play a central role in platelet function. To our surprise, inhibition of Gβγ led to inhibition of in collagen-induced platelet aggregation, which suggests a cross-talk between GPCRs and collagen receptors (Roger et al. 2004), or that Gβγ plays a non-canonical role in platelet activation.

**Inhibition of Gβγ Subunits Reduces Platelet Integrin Activation**

Integrin is a cell adhesion receptor that plays an important role in cell adhesion (aggregation), spreading, retraction, as well as the process of thrombosis (Estevez, Shen, and Du

2015, Karim, Vemana, et al. 2015). Therefore, we next investigated whether inhibition of Gβγ would be associated with a commensurate inhibition of αIIbβ3 activation. Indeed, our data revealed that inhibition of Gβγ inhibits αIIbβ3 integrin activation in platelets treated with gallein in comparison to vehicle. (Figure 3.3A) in response to 5 μmol/L ADP and 5 μg/mL collagen. These results are consistent with the inhibition of aggregation response in gallein treated platelets we observed.

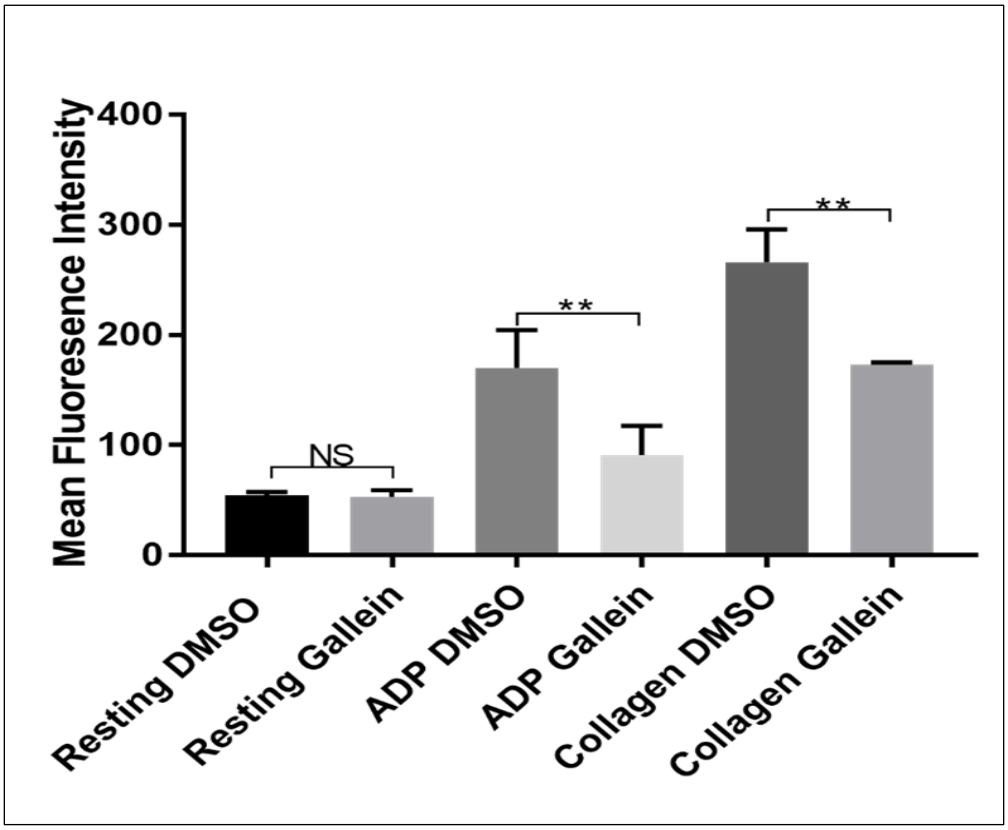



**Figure 3.3: Gallein inhibits αIIbβ3 activation in platelets**. Platelets form healthy human participant treated with gallein or vehicle were incubated with PAC1 antibody, and the fluorescent intensities were measured by flow cytometry after stimulation with 5 μmol/L ADP or 5 μg/ml collagen. Average mean fluorescence intensities shown (**P < 0.01; NS. nonsignificant). The experiment was repeated 3 times, with blood pooled from at least 8 mice each time.

**Inhibition of Gβγ Subunits Alters Hemostasis and Inhibits Thrombosis**

Under our *in vitro* experimental setting, we established that inhibiting Gβγ subunits with small molecular inhibitor gallein significantly inhibits platelet functional responses. Next, we sought to assess if inhibition of Gβγ subunits exerts any effects *in vivo*. Thus, we injected C57BL/6 J mice with small molecular inhibitor (gallein 20 mg/kg twice/day for 3 days). Next, we assessed the impact of inhibition of Gβγ subunits on thrombogenesis, using $FeCl_3$-induced carotid artery thrombosis model, and on hemostasis, a using tail bleeding time assay. We found that mice injected with gallein (20 mg/kg twice/day for 3 days) had a significantly increased occlusion time. In fact, while complete occlusion of the vessel was found to occur shortly after $FeCl_3$ treatment in the control, it was significantly delayed in the gallein injected mice (Figure 3.4A). Similarly, a prolonged tail bleeding time was observed in gallein injected mice, relative to controls (Figure 3.4 B). Taken together, the above data provide evidence- for the first time- that inhibition of the Gβγ subunits not only exerts anti-thrombotic effects, but it also impacts physiological hemostasis.

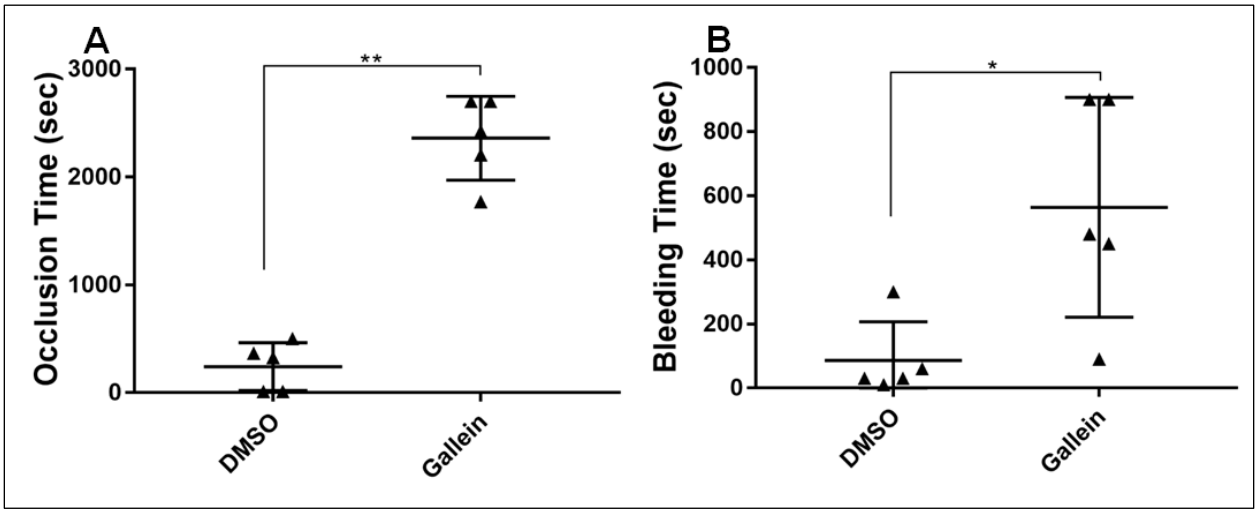



**Figure 3.4**: **Gallein treated mice exhibit prolonged occlusion and bleeding times**. (A) This data illustrates the results of the ferric chloride–induced thrombosis model (as described in the "Methods") comparing gallein and vehicle treated mice time to occlusion. Each point represents the occlusion time of a single animal (DMSO, n=5; and gallein, n=5; **P<0.01). (B) This data illustrates the results of tail bleeding time assay (as described in the "Methods") comparing gallein and vehicle treated mice. Each point represents the tail bleeding time of a single animal (DMSO, n=5; and gallein, n=5; *P<0.05).

**Inhibiting Gβγ Subunits Impairs Clot Retraction and Inhibits Platelet Spreading**

Activation of integrin αIIbβ3 is required for clot retraction and platelet spreading through "outside-in signaling" that triggers an internal signaling cascade. Whether Gβγ subunits play a role in integrin αIIbβ3 signaling cascade is not yet determined. Thus, we sought to investigate Gβγ subunits' role in clot retraction and platelet spreading. Our data shows a defect in clot retraction as well as platelet spreading after inhibition of Gβγ subunits by small molecular inhibitor gallein (Figure 3.5 A, 5B). These data indicate that the Gβγ subunits participate in "outside-in signaling" of platelet.

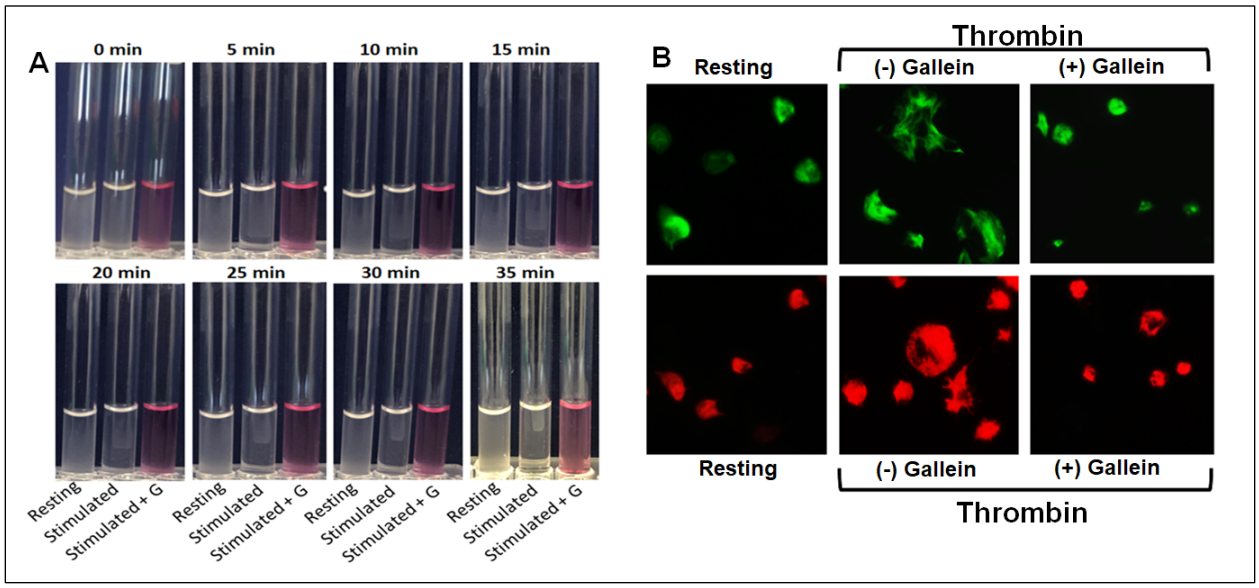



**Figure 3.5: Gallein treatment inhibits clot retraction and platelet spreading.** (A) Whole blood was collected from both gallein and vehicle treated platelets and washed platelets were isolated as discussed in the Methods section. Clot retraction was initiated by adding thrombin (0.1 U/mL) and photographed every 5 minutes for 35 minutes. (B) Platelets from gallein and vehicle–treated platelets were allowed to adhere to fibrinogen-coated coverslips for 5 min after stimulation with thrombin (0.1 U/mL). TRITC Phalloidin and FITC phalloidin were used to stain for F-actin and imaged using Leica DMi8 inverted widefield fluorescence microscope with integrated high precision focus drive. Images were processed using LAS X Wizard imaging software. G: Gallein.

**Discussion & Conclusion**

Herein we show data- some of which for the first time- that inhibiting Gβγ subunits using a small molecular inhibitor- inhibits platelet function *in vitro* as well as alters hemostasis and

inhibits thrombosis *in vivo*. The Gβγ subunits are central players in intracellular signal transduction after GPCRs activation (Smrcka 2013), which make it a viable pharmacological target, especially because GPCRs are the most extensively studied drug targets (Overington, Al-Lazikani, and Hopkins 2006), and their involvement in human diseases is well-known (Hauser et al. 2017). Our results show that inhibiting Gβγ subunits leads to inhibition of agonist- (e.g., ADP-) induced platelet aggregation as well as dense granule secretion. ADP is an important platelet agonist that operates through two GPCRs (P2Y1, P2Y12) (Cattaneo et al. 1990, Hollopeter et al. 2001, Cattaneo and Gachet 1999). ADP receptors are critical for platelet aggregation, thromboxane $A_2$ generation, procoagulant activity, adhesion to immobilized fibrinogen and thrombus formation under shear conditions (Murugappa and Kunapuli 2006).

Advances in studying ADP receptors resulted in the development of drugs like clopidogrel and ticlopidine, which although extensively used in clinical settings, carry many serious side effects (Quinn and Fitzgerald 1999). Targeting Gβγ subunits of the ADP receptors provide more specificity and selectivity in inhibiting platelets activity; this is in part because small molecule inhibitors would not (necessarily) block all GPCR functions. For instance, Gβγ inhibitors do not block activation of Gα subunits by GPCRs, further; Gβγ small molecular inhibitors only block a subset of downstream targets, leaving other process intact (Smrcka 2013). On the contrary, the most commonly used ADP receptor inhibitors cause a complete block of all processes downstream of GPCRs, and in many cases lead to irreversible inhibition (Coukell and Markham 1997, Mills et al. 1992). To this end, these features of inhibiting Gβγ subunits illustrate a new approach to inhibiting platelets with more specificity. To our surprise, targeting Gβγ subunits led to inhibition of collagen-induced platelet aggregation and dense granule secretion. These results might allude to a role for Gβγ subunits in the cross-talk between collagen receptors and GPCRs. Indeed, it has

been long recognized that agonists such as ADP and thromboxane $A_2$ are involved in collagen-induced platelet aggregation to amplify its response (Ardlie, Garrett, and Bell 1986). Moreover, the Gβγ subunits may also play a vital non-canonical role in platelet function, similar to what we have reported with the regulator of G-protein signaling (RGS) proteins (Hernandez et al. 2019, Alshbool et al. 2015, Hensch et al. 2016). The aforementioned effect seen in aggregation and secretion did correspond to reduced activation of integrin αIIbβ3 in platelets treated with gallein. Integrin αIIbβ3 is a major player in platelet function.

To better understand the role of Gβγ subunits in platelets, we sought to investigate whether the effects we observed *in vitro* would be translated to alteration in hemostasis and thrombosis when the platelets are in their natural milieu (*in vivo*). Our results demonstrated and for the first time that inhibiting Gβγ subunits modulates hemostasis – increases tail bleeding time – and inhibits arterial thrombus formation– increases time to occlusion. These findings are consistent with the observed *in vitro* phenotype and support the initial hypothesis that the Gβγ subunits do indeed serve as modulators of platelet function, hemostasis, and thrombogenesis.

After being activated, platelets transform and flatten themselves to increase their surface contact area at the site of a vascular injury by maneuvering their plasma membrane. This process is called platelet spreading and involves outside-in signaling of αIIbβ3(Lee et al. 2012). This shape change or spreading of activated platelets is an essential step for normal hemostasis, as well as thrombogenesis (Furie and Furie 2008). Our findings show that in the presence of the Gβγ subunits inhibitor, platelet spreading was dramatically reduced. In the same context, we also observed inhibition of clot retraction in platelets treated with the Gβγ subunits inhibitor. These findings suggest that Gβγ subunits play a role in outside-in-signaling of integrin αIIbβ3. To this end, studies have shown that Gβγ subunits regulate small G-proteins such as Rho and Rac that have impact on

platelet shape change and clot retraction (Chari-Turaga and Naik 2011, Cervantes-Villagrana et al. 2019).

In summary, our results provide evidence that pharmacological inhibition/targeting of Gβγ subunits reduces platelet function, both *in vitro* and *in vivo*. Consequently, Gβγ subunits have the potential to serve as a target to develop a therapeutic agent that can be added to the available interventions to manage thrombotic disorders. Targeting Gβγ subunits might be advantageous because it is potentially more selective(Smrcka 2013) compared to antiplatelet agents that target GPCRs. Future studies are warranted to investigate these notions.

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# Glossary

| | |
|---|---|
| ADP | Adenosine diphosphate |
| AKT | Protein kinase B |
| BDNF | Brain-derived neutrophic factor |
| BP | Blood pressure |
| CVD | Cardiovascular disease |
| CAD | Coronary artery disease |
| CEA | Carcinoembryonic antigen |
| CO | Carbon monoxide |
| DNA | Deoxyribonucleic acid |
| ENDS | Electronic nicotine delivery systems |
| ERK | Extracellular signal regulated kinases |
| EPA | Environmental Protection Agency |
| FDA | Food and Drug Administration |
| FEF | Forced expiratory flow |
| FeNO | Fractional exhaled nitric oxide |
| FEV | Forced expiratory volume |
| FMD | Flow-mediated dilation |
| FVC | Forced vital capacity |
| GPCRs | G-protein coupled receptors |
| GSH | Glutathione |
| HR | Heart rate |
| HCT | Hematocrit |
| IHD | Ischemic heart disease |
| IL | Interleukin |
| MPV | Mean platelet volume |
| MI | Myocardial infarction |
| mRNA | Messenger RNA |
| NO | Nitric oxide |
| PS | Phosphatidylserine |
| PAHs | Polycyclic aromatic hydrocarbons |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |
| TXB2 | Thromboxane B2 |
| TNF | Tumor necrosis factor |
| WPS | Waterpipe smoke |
| VEGF | Vascular endothelium growth factor |

# Vita

Ahmed Alarabi was born and raised in Tripoli, Libya. He obtained his MD from Tripoli medical school in 2005. Between 2005 and 2008, Ahmed worked as senior house officer in the surgical department in Tripoli medical center, the busiest hospital in Tripoli. In 2008 he was awarded a scholarship to study occupational medicine and health at The university of Pretoria, South Africa and graduated with a diploma in occupational medicine and health in 2009. After graduation, he resumed working as a physician for different multinational organizations to manage health of employees of these organizations, and he focused on work related diseases and he started developing interest in the area of public health and started applying and running locally funded health programs to monitor employees cardiovascular health and help them engage into healthy initiatives, such as quitting smoking through smoking stoppage campaign. In 2015, Ahmed was awarded Fulbright scholarship to study public health in the United States at the University of Southern Mississippi. After graduation in 2017, he wanted to complete his graduate education and he was admitted to the Interdisciplinary Health Sciences PhD program at the University of Texas at El Paso. In the program Ahmed began perusing his third degree in Interdisciplinary Health Sciences, and he joined Dr. Fadi Khasawneh's (adviser) and Dr. Fatima Alshbool's (co-adviser) platelets laboratory where he developed his passion to link basic science to major public health issues. He investigated the impact of smoking Hookah on cardiovascular health in the context of blood platelets. And he also illustrated and for the first time the importance of the betagamma subunits in platelet function, which might pave the way for therapeutic targeting of this protein to treat thrombotic cardiovascular disease.

Contact Information: Ahmed.Alarabi.lihpsm@gamil.com
This thesis/dissertation was typed by Ahmed Alarabi