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Platelet Biology And Thrombosis: Juul E-Cigarette Exposure And 5ht2a Receptor Drug Discovery

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Dedication

For my loving family who has always been there for me. You have been an immense motivator to me. My hardworking father who has always provided me with opportunities and guided me along the way. My diligent mother has always taken care of me and who taught me respect, honesty, compassion, and perseverance. My brothers who have always been by my side guiding and helping me. I dedicate this work to everyone who has been with me in this endeavor.

PLATELET BIOLOGY AND THROMBOSIS: JUUL E-CIGARETTE EXPOSURE AND $5HT_{2A}$ RECEPTOR DRUG DISCOVERY

by

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CHAPTER I: Platelet biology and E-Cigarette

Section 1.1. Platelet physiology and function

Cardiovascular disease annually accounts for 17 million deaths worldwide. It is the number one cause of death in the world (1). According to the American Heart Association, annually, one in every three deaths are caused from a heart disease, stroke and other cardiovascular diseases in the United States alone (2). Cardiovascular disease is tightly linked to platelets, as platelets play an important role in the development of thrombosis, which is a form of and a risk factor for other types of cardiovascular diseases (3).

Platelets are tiny disk-shaped anucleate cell fragments that are created in the bone marrow from megakaryocytes (4-6). Platelets primarily function as regulators of hemostasis and thrombosis (7). Hemostasis is the spontaneous arrest of blood loss from an injured blood vessel (3, 6). In contrast, thrombosis is the pathological process in which an occlusive thrombus forms and blocks blood flow to vital organs (i.e. inappropriate formation of clots) (3).

Section 1.1.1. Platelet clot formation

When injury to the blood vessel wall occurs, two adhesion receptors are responsible for regulating initial platelet adhesion, namely glycoprotein Ib-IX-V which binds to von Willebrand factor, and glycoprotein VI which binds to collagen (8-13). Subsequently, rapid signal transduction causes platelets to activate. During this activation; spreading and secretion occur while platelets also undergo a cytoskeletal change, thus, changing shape (7). In addition, aggregation and adhesion is supported by inside-out activation of integrins. The main platelet integrin that is activated during platelet aggregation is αIIbβ3

(also known as GPIIb/IIIa), which binds to fibrinogen to mediate platelet aggregation (12, 14, 15). Furthermore, when platelet activation occurs, platelet secretion is initiated ultimately resulting in the release of different platelet agonists, such as, adenosine diphosphate (ADP) from the platelets storage granules, as well as the synthesis of the lipid thromboxane A_2 (TXA_2). These agonists will lead to activation of further platelets and their recruitment, until a plug made of platelet aggregates finally forms at the injury site (4, 7, 16). In addition, platelets are involved in the conversion of the clotting factor prothrombin to thrombin. Thrombin will convert fibrinogen to fibrin. Fibrin will create a mesh in and around the injury site, thus, completing the hemostatic process (4).

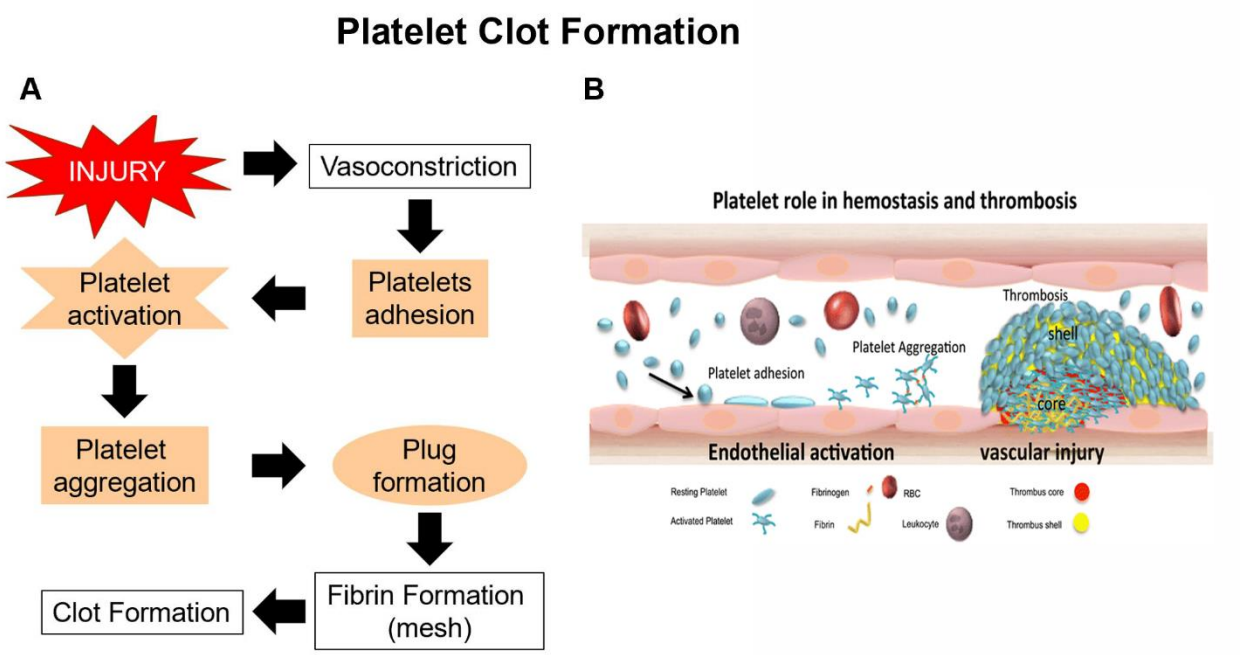

Figure 1.1. (A) Schematic representation of platelet clot formation. (B) Platelet role in hemostasis and thrombosis (17).

Section 1.1.2. Transmembrane receptors

Agonists receptors that belong to this family are expressed on the surface of the platelets. These agonists include ADP and thrombin receptors which play an important role in platelet aggregation and secretion. ADP is a weak agonist that is found in dense granules, and is secreted from platelets upon activation (16). ADP receptors are separated into two different G-Protein coupled receptors (GPCR), those being $P2Y_1$ and $P2Y_{12}$; both of which engage a different signaling pathway, namely $G\alpha_q$ and G_i, respectively (9, 13, 16, 18, 19).

In contrast, thrombin is a strong agonist that is produced from the enzymatic cleavage of prothrombin (20). Further, thrombin receptors are separated into two different GPCR's (in humans) which are identified as PAR-1 and PAR-4, and they each engage a different signaling pathway: $G\alpha_q$, $G\alpha_i$, $G\alpha_{12/13}$ and $G\alpha_q$, $G\alpha_{12/13}$, respectively (9, 13, 18).

Section 1.1.3. G-protein coupled receptors signaling

The GPCRs are composed of a single polypeptide that is folded into a double helix of seven segments, which is transmembrane in nature, and they are coupled to heterotrimeric guanine-nucleotide binding-proteins (G-proteins), composed of an α, β, and γ subunits, belonging to the G_q, $G_{12/13}$, and G_i families. Upon ligand binding, the receptor allows an exchange of GDP for GTP in the α-subunit. The exposed α-subunit, in case of G_q for example activates an effector, "β isoform of phospholipase C" (PLC- β), which cleaves the membranous phospholipid "Phosphatidylinositol biphosphate" (PIP_2), thus, separating them into Inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 will then bind into its receptor in the endoplasmic reticulum, which will raise the levels of Ca^{2+} while simultaneously decrease cyclic adenosine-3',5'-monophosphate (cAMP).

Following, Ca^{2+} calmodulin will bind to calmodulin-dependent protein kinase and start protein phosphorylation. Similarly, DAG will bind to protein kinase C and activate protein phosphorylation. The GTPase activity of the α-subunit promotes reassociation with βγ to terminate ligand induced signaling (9, 13, 18, 21-25).

The function of each agonist is dependent on the specific G-protein subunit i.e. Gαq's effector-as describe above- is PLC-β which increases IP_3 (inositol-1,4,5-trisphosphate) / DAG. Similarly, Gβγ effector is PLC-β and PI3K-γ which increase IP_3/DAG and 3-PPIs, respectively (13). $G\alpha_{12/13}$ effector is p115-RhoGEF whose function is actin cytoskeleton reorganization. $G\alpha_s$ and $G\alpha_i$ effector is adenylate cyclase, and upon activation of their corresponding GPCR, the cyclic adenosine monophosphate (cAMP) levels increase or decrease, respectively (9, 13, 18).

Other platelet agonists bind to their own GPCR; e.g. TXA_2 binds to the thromboxane receptor, which couples to G_q and $G_{12/13}$ (13, 18), whereas 5-HT binds to 5-HT_{2A} Receptor, which couples with G_q (26).

Section 1.2. E-cigarettes

Electronic cigarettes/e-cigarettes are electronic nicotine delivering systems (ENDS). The e-cigarette user generates a mixture of vapor (derives from flavored or non-flavored liquids) and nicotine that is inhaled in the form of aerosol (27). The nicotine containing or free liquid normally utilized in e-cigarettes is commonly known as e-liquid. For certain types of e-cigarette devices (e.g., JUUL), the e-liquid is found in pods that are used in the vaping process. Often, e-liquids are composed of flavoring, nicotine, glycerin, and propylene glycol (27). Even with all existing different designs of e-cigarettes, e-cigarettes

share common functions and compositions i.e. a cartridge (filled with e-liquid), a rechargeable lithium battery, and a vaporization chamber (27, 28). Usually, the e-cigarette device has a sensor in the mouth piece, which will activate the atomizer when there is airflow (27, 28). Further, the atomizer vaporizes the e-liquid in the vaporizing chamber and delivers the aerosol mixture to the lungs (27, 28).

Currently, there are first, second, third, and fourth generation of e-cigarettes devices. First-generation e-cigarettes look like a traditional cigarette with no refillable cartridge and a low voltage (3.7 volts) (28, 29). Due to its low voltage output and low nicotine delivery, new users are more susceptible to use this device. Often, rechargeable and disposable e-cigarettes are considered first-generation e-cigarettes (28, 30). Smaller devices like the first-generation e-cigarettes had low battery life. Similarly, second-generation e-cigarettes still keep a somewhat traditional cigarette look, but now with adjustable voltage (3-6 volts), high tank capacity, and refillable cartridges (28, 30, 31). Due to its high voltage output, users can inhale higher puff volumes compared to the first-generation. In contrast, third-generation e-cigarettes now have a bulky look and are heavy, with refillable cartridges, longer battery life/size and adjustable voltage (28, 30). Fourth-generation devices can control temperature and airflow. Users utilizing fourth-generation devices can inhale serious amounts of e-liquid per use (28).

Section 1.2.1. E-cigarette JUUL

With popular demand, the e-cigarette market has kept increasing, thus, new devices continue to be manufactured. Recently, the JUUL e-cigarette brand has attained wide popularity among smokers, non-smokers, and especially the youth (32-34). The JUUL e-cigarette device is compact, low weight, user friendly, has good battery life, and creates

a small vapor cloud (35, 36). The JUUL e-liquid is often called the JUUL pod. Each pod contains ~0.7ml with 3% or 5% nicotine by weight. According to JUUL labs, each JUUL pod is composed of glycerol, propylene glycol, flavors, nicotine, and benzoic acid (37).

Currently, the JUUL e-cigarette is marketed as an e-cigarette device with a higher safety profile than traditional cigarettes (38). However, this statement is not evidence based and to our knowledge, there is a lack of information and studies regarding the safety profile of the JUUL e-cigarette device in the context of cardiovascular system. The state of regulatory laws (or lack of) for the JUUL e-cigarette is alarming as new young users continue to use this device (32-34). To this end, it is of great importance to investigate and establish the safety profile of JUUL e-cigarette, to not only educate the public but to also inform new safety/ regulatory laws.

Section 1.3.1. Serotonin

Serotonin (5-Hydroxytripamine or 5-HT) is a weak agonist to platelet aggregation, yet, is an important regulator of hemostasis and thrombosis (39). Serotonin is found in the platelets granules and is released upon platelet activation (39). When released, serotonin induces vasoconstriction via modulation of endothelial cells and nitric oxide(39). Serotonin is also known to enhance platelet aggregation in the presence of other agonists like ADP (40-42). For normal hemostasis, proper platelet activation and recruitment is necessary. Improper recruitment and excessive aggregability of platelets can lead to thrombosis and thromboembolism (43-46).

The 5-HT_{2A} receptor (5-HT_{2A}R), a subtype of $5HT_2$, plays an important role in platelet function (47). In addition, 5-HT_{2A}R is also a GPCR (48), and is involved in the

development of different kinds of cardiovascular disorders (49, 50). Even though there is much information known about $5HT_{2A}R$ in pathogenesis of thrombotic disease (41, 49-54), there are no antagonists currently available for thrombosis/platelet related clinical use which target this specific pathway (53-55). Thus, it is vital to understand the structural biology of $5HT_{2A}R$ and signaling transduction mechanisms to develop a drug that can modulate $5HT_{2A}R$ activation.

CHAPTER II: JUUL E-Cigarette Increases the Risk of Thrombosis and Enhances Platelet Function in Mice.

Section 2.1. Abstract

Background: Smoking is the main preventable cause of death in United States and is associated with serious cardiovascular health consequences, including thrombosis disease. In recent years, electronic-cigarettes (i.e. e-cigarettes) have attained wide popularity among smokers, non-smokers, pregnant females, and even youth. One particular e-cigarette that has gained popularity over the past 2 years is the JUUL. JUUL labs has grown expeditiously to the point of attaining $150 million US dollars in retail sales, which accounts for 40% of the e-cigarette retail market share. JUUL's popularity is alarming; especially among youth. Interestingly, there is no information/studies regarding the effect of JUUL on cardiovascular diseases, specifically in the context of modulation of platelet activation. Thus, it is important to discern the cardiovascular disease health risks associated with JUUL. **Methods:** We utilized a passive e-vapeTM vapor inhalator system where C57BL/6J mice (10-12 weeks old) were exposed to JUUL e-cigarette vape/smoke. Menthol flavored JUUL pods containing 5% nicotine by weight were used as the e-liquid. Mice were exposed to a total of 70 puffs per day for 2 weeks; 3 secs puff duration, 25 secs puff interval, and 50 ml puff volume. We analyzed the effects of JUUL exposed mice relative to those exposed to clean air, in the context of platelet function *in vitro* and thrombogenesis *in vivo*. **Results:** Our results indicate that short-term exposure to JUUL e-cigarette causes hyper-activation of platelets *in vitro* and shortens the thrombus occlusion as well as hemostasis/bleeding time. **Conclusion**: Our findings show for the first time that short-term exposure to JUUL E-cigarette can increase the risk of

thrombogenic events and can alter physiological hemostasis; in part by modulating platelet function; in mice.

Section 2.2. Introduction

It is well established that tobacco smoking is associated with cardiovascular disease/CVD, which is the leading cause of death in the world (1, 56, 57). Being the single most preventable risk factor for the development of CVD (57, 58), traditional cigarette smoking/usage has declined and new products have been introduced into the market (59-62). To this end, electronic-cigarettes (e-cigarettes) have attained wide popularity in recent years, due to the perception of being a safer option/alternative relative to tobacco smoking, with minimal or no harm (38). As the e-cigarette market keeps growing at a fast pace, hundreds of devices/models continue to be invented. One particular e-cigarette device that has gained significant popularity over the past 2 years is known as “JUUL” (32-34). JUUL sales have grown expeditiously to the point of attaining $150 million US dollars in retail sales, which accounts for 40% of the e-cigarette retail market share (63). It has a novel slim USB-like design that makes it easy to use and carry, and it also comes with prefilled e-liquid pods and is available in various flavors (64, 65). The unique design and appealing nature of the device and flavors along with the advertisement/marketing campaign attracts smokers, non-smokers, and even the youth (35, 36, 66). Of particular concern is the fact that the youth has the highest increase in e-cigarettes usage (67, 68), especially the JUUL device (65, 69-72). Thus, over 3.6 million kids have used e-cigarettes in 2018, which corresponds to 78% and 48% increase in usage among high school and middle school students, respectively, compared to 2017 (67, 68, 73). In fact, the Food and Drug Administration (FDA) raised concerns regarding JUUL’s popularity in young

communities and the potential for creating a new generation of smokers (38, 74). Based on these considerations, it is paramount to establish the safety of exposure to JUUL e-cigarette, including in the context of cardiovascular disease. To this end, limited studies have evaluated the impact of traditional e-cigarettes on the genesis of thrombosis-based CVD. Nonetheless, we have previously shown that short-term e-cigarette exposure can alter physiological hemostasis and increase the risk of thrombosis, via modulating platelet function in mice (75). Additionally, a recent crossover study that was conducted on tobacco smokers and nonsmokers found that acute use of e-cigarettes enhanced platelet aggregation (76). To our knowledge, there is no information regarding the impact of JUUL e-cigarette on the cardiovascular system.

JUUL has certain/unique features and does not resemble the traditional e-cigarette as the device is circuit and temperature controlled and generates a small cloud. Furthermore, the e-liquid composition is also different, as it contains a nicotine salt and higher concentrations of nicotine (3% and 5% by volume) (64, 77). According to JUUL labs, each e-liquid JUUL pod is intended to give 200 puffs, which equals one pack of combustible cigarettes in terms of the delivered nicotine concentration (78). Furthermore, the use of the salt results in higher amount of nicotine being delivered to users. To this end, a recent study did in fact show that youth are exposed to higher concentrations of nicotine when they use JUUL, compared to combustible cigarettes (64, 79). Therefore, we characterized the impact of JUUL exposure on thrombogenesis and platelet function- in mice- utilizing a whole-body exposure protocol that mimics real life JUUL users. Our findings demonstrate that two weeks exposure to JUUL e-cigarette increases the risk of thrombosis and enhances hemostasis, in mice. In addition, JUUL exposure enhances

platelet activity as reflected by enhanced aggregation, secretion, and integrin GPIIb/IIIa activation. These findings indicate that the JUUL e-cigarette is not safe as people believe. Further studies are needed to shed more light on its harmful effects to the cardiovascular health.

Section 2.3. Materials and Methods

Section 2.3.1. Materials and Reagents

JUUL device and JUUL Pods (5% nicotine and menthol flavoring) were purchased from the JUUL Labs, Inc. (www.juul.com). Stir bars and other disposables were purchased from Chrono-Log Corporation (Havertown, PA). ADP and thrombin were purchased from Sigma Aldrich (St. Louis, MO). Fluorescein isothiocyanate (FITC)-conjugated anti-P-selectin and FITC-conjugated Annexin V were purchased from Cell Signaling Technology, Inc (Danvers, MA). Phycoerythrin (PE)-conjugated JON/A antibody was obtained from Emfret analytics (Würzburg, Germany). The Mouse/Rat Cotinine ELISA kit was from Calbiotech (El Cajon, CA). Platelet count was determined using an automated hematology analyzer (Drew Scientific Dallas, TX). The primary antibodies (ERK, pERK, Akt, and pAkt) were purchased from cell signaling Technology, Inc. (Danvers, MA). Other reagents were of analytical grade.

Section 2.3.2. Animals

C57BL/6J (10-12-week-old male) mice were obtained from Jackson Laboratory (Bar Harbor, ME). Mice were housed in groups of 1–5 at 24 ° C, under 12/12 light/dark cycles, with access to water and food ad libitum. All animal experimental protocols were approved

by the Institutional Animal Care and Use Committee (IACUC) of The University of Texas at El Paso.

Section 2.3.3. JUUL E-cigarette Exposure Protocol

C57BL/6 mice (10-12 weeks old males) were exposed to a total of 70 puffs per day for a period of 2 weeks. Puff duration was 3 seconds (puff volume was 50 ml) with puff intervals of 25 seconds. These conditions mimic real-life exposure scenarios (75, 80-82). As for the control mice, they are matched in terms of age and sex to the JUUL e-cigarette exposed mice and were exposed to clean air. The JUUL e-cigarette exposed and control mice were deprived of food and water during exposure. The apparatus used to generate the aerosol (e-vape) is a custom-made bench-top e-VapeTM vapor inhalation system for rodents (La Jolla Alcohol Research, Inc). The system has four chambers each connected to its own “mode box”. The “mode box” is custom made with a USB outlet to be compatible with the JUUL device, and is connected to a controller that allows us to automatically pre-determine the following experimental conditions: the puff time/duration, puff interval (time between puffs), and/or number of puffs. In terms of the JUUL pods, each contained 0.7 mL of e-liquid with 5% nicotine by weight and menthol flavoring (32, 83).

Section 2.3.4. Methods

The data, analytic methods, and study materials will be made available to other researchers for purposes of reproducing the results or replicating the procedure. Because there are no specific study materials per se, requests for data, and the vaping protocol will be honored on a case-by-case basis.

Section 2.3.5. Cotinine Assay

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

Section 2.3.6. *In vivo* Thrombosis Model

JUUL e-cigarette and clean air exposed mice were anesthetized by IP injections of avertin. Then, the left carotid artery was exposed and cleaned, and baseline carotid artery blood flow was measured with Transonic micro-flowprobe (0.5 mm, Transonic Systems Inc., Ithaca, NY). After stabilization of blood flow, 7.5% ferric chloride ($FeCl_3$) was applied to a filter paper disc (1-mm diameter) that was immediately placed on top of the artery for 3 min. Blood flow was continuously monitored for 30 min, or until blood flow reached stable occlusion (zero blood flow for 2 min). Data was recorded and time to vessel occlusion was calculated as the difference in time between stable occlusion and removal of the filter paper (with $FeCl_3$). An occlusion time of 30 min was considered as the cut-off time for the purpose of statistical analysis.

Section 2.3.7. Tail Bleeding Time Assay

Hemostasis was examined using the tail transection technique. Mice were anesthetized and placed on a 37 °C homeothermic blanket. 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 cessation was measured. Bleeding time of 15 min was considered as the cut-off time for the purpose of statistical analysis.

Section 2.3.8. Murine Platelet-Rich Plasma Preparation

Clean air– or JUUL e-cigarette–exposed mice were anesthetized, and blood was collected from the heart. Coagulation was inhibited by 0.38% sodium citrate solution (Fisher Scientific, Hampton, NH). Blood was centrifuged (237g for 15 minutes) at room temperature, and the platelet-rich plasma was then collected. Platelets were counted with the HEMAVET® and their count adjusted to 7 $x10^7$ platelets/mL before each experiment.

Section 2.3.9. Washed Platelet Preparation

Washed platelets were prepared as described previously (21, 84, 85). Blood was drawn from C57BL/6J mice (10–12 weeks old) as discussed above and mixed with phosphate-buffered saline, pH 7.4, incubated with PGI_2 (10 ng/mL; 5 minutes), followed by centrifugation at 237x g for 10 minutes at room temperature. Platelet-rich plasma was recovered, and platelets were pelleted at 483x 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 Dglucose) 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) without EGTA, apyrase, or PGI_2. Platelets were counted using the HEMAVET® and adjusted to the indicated concentrations.

Section 2.3.10. *In vitro* Platelet Aggregation

Platelet-rich plasma from clean air– or JUUL e-cigarette–exposed mice was activated with 1 μM ADP or 0.1 U/ml thrombin. Platelet aggregation was measured by the

turbidometric method using a model 700 aggregometer (Chrono-Log Corporation). Each experiment was repeated at least 3 times, with blood pooled from 5-8 mice each time.

Section 2.3.11. Flow Cytometric Analysis

Flow cytometric analysis was performed as previously prescribed (21). Washed platelets were stimulated with ADP (1 μM), or thrombin (0.1 U/ml). The reactions were stopped by fixing the platelets with 2% formaldehyde for 30 min at room temperature. Finally, platelets were incubated with FITC-conjugated Annexin V and anti– P-selectin, or Jon/A PE-conjugated antibodies at room temperature for 30 min in the dark. Finally, the platelets were diluted 2.5-fold with HEPES/ Tyrode buffer (pH 7.4). The samples were transferred to FACStubes and fluorescent intensities were measured using a BD Accuri C6 flow cytometer and analyzed using CFlow Plus (BD Biosciences, Franklin Lakes, NJ).

Section 2.3.12. Immunoblotting

Immunoblot was carried out as described before (85). Briefly, clean air– or JUUL–exposed washed platelets were stimulated with ADP (1 μM) or 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 anti-rabbit or anti-mouse 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 with Image Lab software Version 4.1 (Bio-Rad).

Section 2.3.13. Statistical Analysis

All experiments were performed at least three times. Analysis of the data was performed using GraphPad PRISM statistical software (San Diego, CA) and presented as mean ± SD. The Mann-Whitney test was used for the evaluation of differences in mean occlusion and bleeding times. Analysis was also conducted using the t-test and/or one-way ANOVA with Tukey's multiple regression comparison as post hoc were used as applicable. Significance was accepted at $P<0.05$, unless stated otherwise.

Section 2.4. Results

Section 2.4.1. JUUL E-Cigarette Exposure Systematically Delivers Nicotine

In order to verify if our whole-body exposure model does systematically deliver nicotine to the mice, cotinine was measured due to the fact that it is a major metabolite of nicotine, thus serving as a marker of nicotine delivery (86, 87). Therefore, urinary cotinine levels were measured using ELISA, and our data showed significant levels of cotinine in the urine of JUUL e-cigarette exposed mice, whereas it was undetectable in the clean air control mice (Fig. 2.1.).

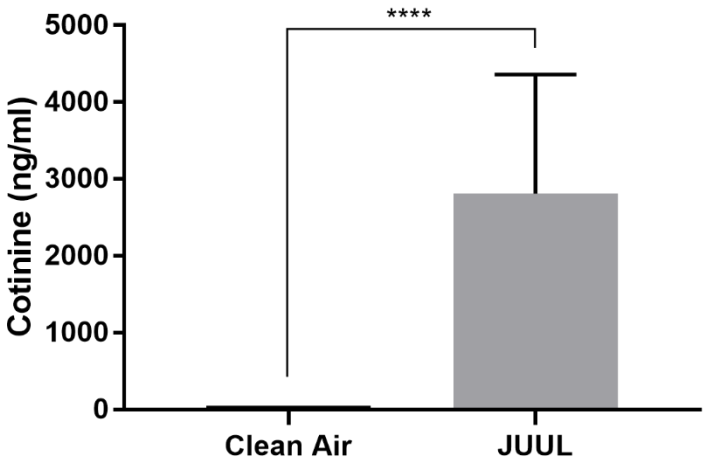

Figure 2.1. JUUL exposure results in systemic delivery of nicotine (cotinine). Urinary cotinine levels from JUUL and clean air exposed mice (clean air, n = 5; and JUUL, n = 5; ****P<0.0001).

Section 2.4.2. JUUL E-Cigarette Exposed Mice Exhibit Enhanced Physiological Hemostasis and Thrombus Formation

In terms of characterizing the negative CV effects of the JUUL e-cigarette, we initially investigated its capacity to modulate physiological hemostasis by utilizing the tail bleeding time assay. Our findings show that JUUL-exposed mice have significantly shortened tail bleeding time compared to mice exposed to clean air (Fig. 2.2.A). Next, we examined the thrombotic effect of JUUL e-cigarette exposure by implementing the carotid artery injury-induced thrombosis model. We observed that JUUL-exposed mice have remarkably shortened occlusion time compared to their controls (Fig. 2.2.B). These results indicate that JUUL e-cigarette exposure increases the risk of thrombosis and enhances physiological hemostasis.

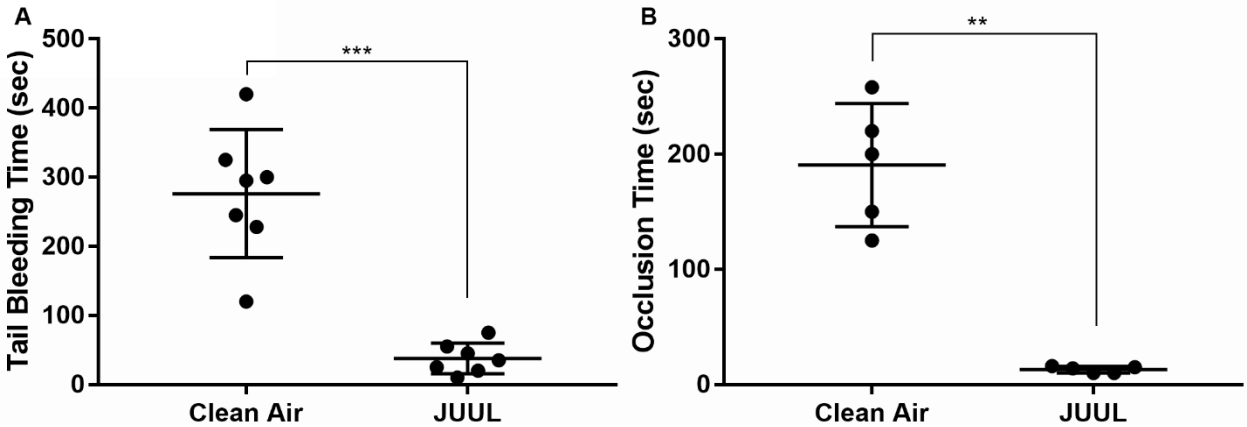

Figure 2.2. JUUL exposure shortens the tail bleeding and occlusion times. (A) JUUL e-cigarette and clean air exposed mice were subjected to the tail bleeding time assessment. Each point represents the tail bleeding time of a single animal (clean air, n = 7; and JUUL, n = 7; ***P<0.001). (B) JUUL e-cigarette and clean air exposed mice were

subjected to the ferric chloride-induced thrombosis model. Each point represents the occlusion time of a single animal (clean air, n = 5; and JUUL e-cigarette, n = 5; **P<0.01).

Section 2.4.3. JUUL E-Cigarette Exposure Enhances ADP-, and Thrombin-Induced Platelet Aggregation

We have previously shown that e-cigarette exposure modulates platelet function (75). Thus, we sought to investigate whether the JUUL e-cigarette exposure would also produce platelet effects. Consequently, we assessed the effects of JUUL e-cigarette exposure on platelet aggregation induced by “two” separate G-protein coupled receptors, namely those activated by ADP and thrombin. Our data revealed that exposure to JUUL e-cigarettes results in higher ADP-induced platelet aggregation, in comparison to clean air control platelets (Fig. 2.3.A). Similarly, thrombin-induced platelet aggregation was also elevated/enhanced in the JUUL e-cigarette exposed platelets (Fig. 2.3.B). These results suggest that JUUL e-cigarette exposure can lead to platelet hyperactivity.

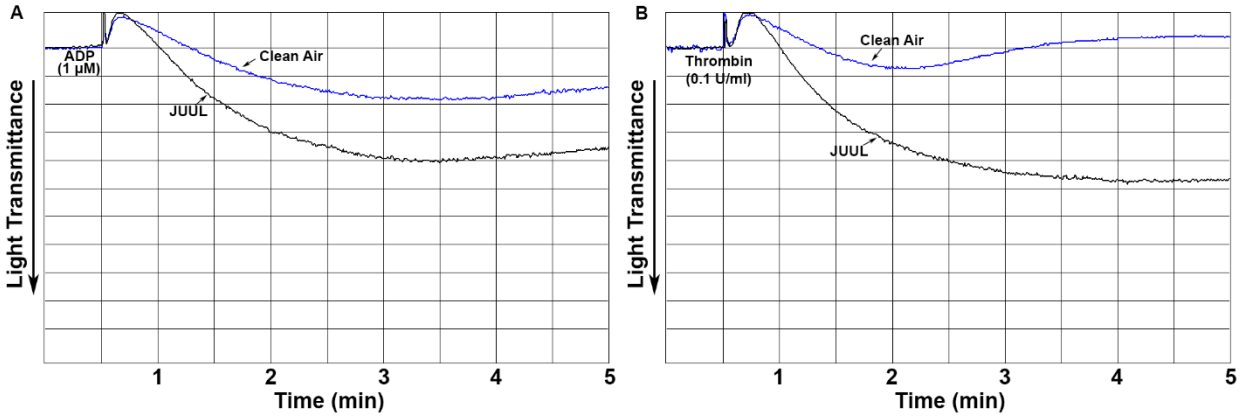

Figure 2.3. Platelet aggregation is enhanced in JUUL exposed mice. Platelets from JUUL e-cigarette and clean air exposed mice were stimulated with (A) ADP (1 µmol/L), or (B) thrombin (0.1 U/ml). The aggregation response was measured using an

aggregometer. Each experiment was repeated 3 times, with blood pooled from 5-8 mice each time.

Section 2.4.4. JUUL E-Cigarette Exposure Enhances ADP-, and Thrombin-Induced Platelet Secretion

Agonist-induced exocytosis or granules release plays a very important role in the amplification of initial platelet activation during primary hemostasis (88-90). Accordingly, we determined if JUUL e-cigarette exposure exerts any impact on dense and α granule secretion. In line with our aggregation data, we observed enhanced ATP secretion from platelets obtained from the JUUL e-cigarette exposed mice in response to ADP (Fig. 2.4.A) and thrombin (Fig. 2.4.B) agonist stimulation, when compared to platelets from the clean air exposed mice. Moreover, JUUL e-cigarette exposed platelets had significantly higher ADP-, and thrombin-triggered P-selectin expression (Fig. 2.4.C).

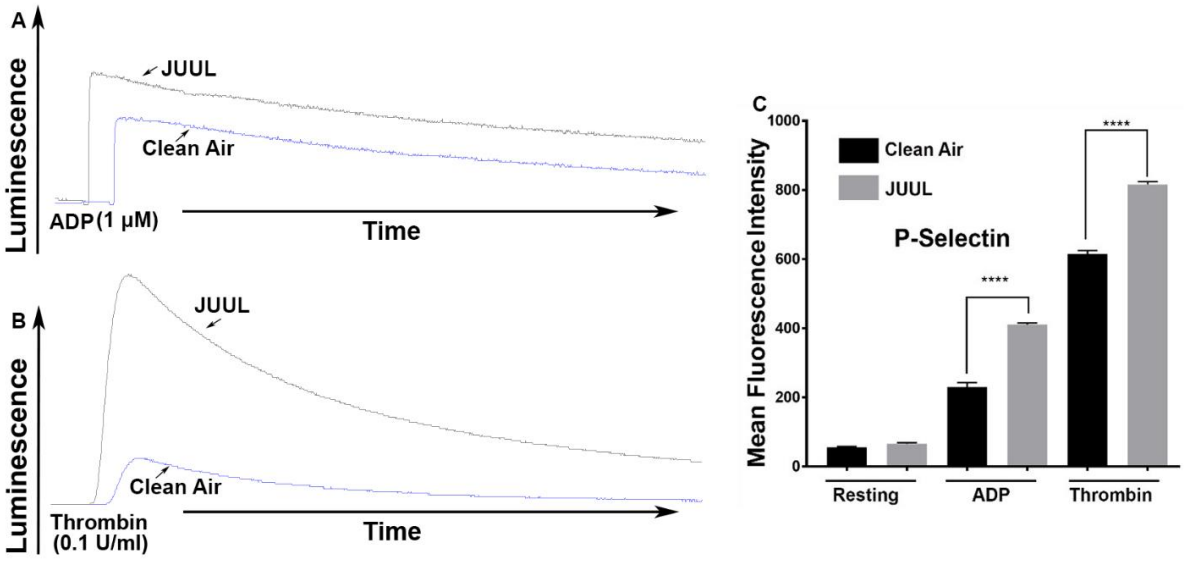

Figure 2.4. Platelet dense and α granule secretion are enhanced in JUUL exposed mice. Platelets from JUUL e-cigarette and clean air exposed mice were incubated with

luciferase luciferin (12.5 µL), then were stimulated with (A) ADP (1 µmol/L), or (B) thrombin (0.1 U/ml). ATP release (dense granules) was detected as luminescence and measured by a lumi aggregometer. (C) Platelets from JUUL e-cigarette and clean air exposed mice were washed, before stimulation with 1 µmol/L ADP, or 0.1 U/ml thrombin. Platelets were incubated with fluorescein isothiocyanate conjugated CD62P antibody, and the fluorescent intensities were measured by flow cytometry. The average mean fluorescence intensities are showed (****P<0.0001). Each experiment was repeated 3 times, with blood pooled from 5-8 mice each time.

Section 2.4.5. JUUL E-Cigarette Exposure Enhances Agonist-Induced Integrin GPIIb/IIIa Activation and Phosphatidylserine Exposure

We next investigated if there is a commensurate increase in integrin GPIIb/IIa (αIIbβ3) activation in the JUUL e-cigarette exposed platelets, given their increased aggregation phenotype (91, 92). We found that JUUL e-cigarette exposed platelets had an enhanced agonist-mediated activation of GPIIb/IIa, when compared to clean air exposed platelets (Fig. 2.5.A). Moreover, agonist-mediated phosphatidylserine (PS) expression, which is a critical element for efficient activation of the coagulation cascade and essential aspect of clot formation, was also found to be enhanced in the JUUL platelets (Fig. 2.5.B).

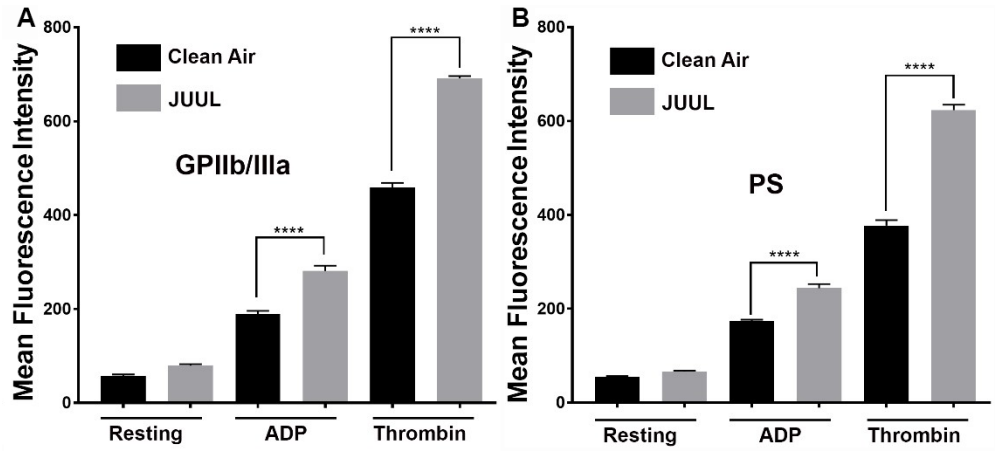

Figure 2.5. Integrin GPIIb/IIIa activation and phosphatidylserine (PS) exposure are increased in JUUL exposed mice. Platelets from JUUL e-cigarette and clean air exposed mice were washed, before stimulation with 1 μmol/L ADP, or 0.1 U/ml thrombin. Platelets were incubated with phycoerythrin-conjugated JON/A antibody, and the fluorescent intensities were measured by flow cytometric analysis (A); (*****P<0.0001). Platelets were incubated with fluorescein isothiocyanate–conjugated Annexin V antibody, and the fluorescent intensities were measured by flow cytometric analysis (B). Average mean fluorescence intensities shown (****P<0.0001). Each experiment was repeated 3 times with blood collected from 5-8 mice each time.

Section 2.4.6. JUUL E-Cigarette Exposure Enhances Akt and ERK Activation

Finally, and in light of the functional data indicating platelet hyperactivity, we sought to determine if biochemical markers of platelet activation would also be elevated as a result of JUUL e-cigarette exposure. Indeed, Akt and ERK phosphorylation were found to be elevated in the JUUL platelets, in response to ADP (1 μM; Fig. 2.6.).

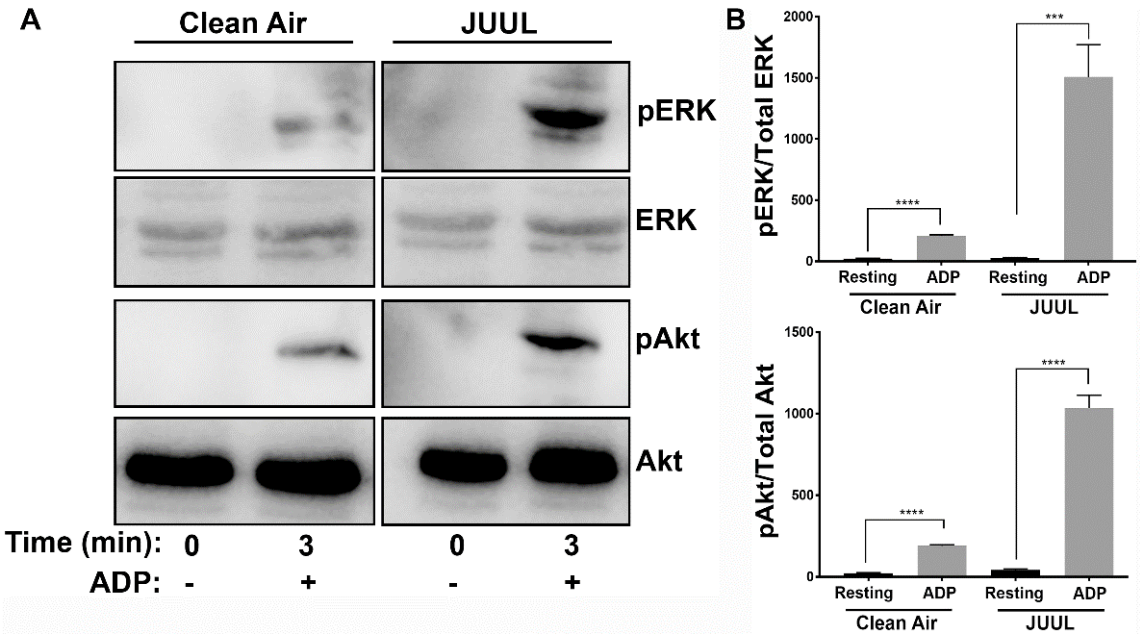

Figure 2.6. Platelet Akt (protein kinase B) and ERK (extracellular signal-regulated kinases) activation (phosphorylation) is enhanced in JUUL exposed mice. Platelets from JUUL and clean air–exposed mice were prepared and washed. (A) Platelets were stimulated with 1 μmol/L adenosine diphosphate (ADP) for 3 min, and proteins were lysed using 1×sample buffer. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis before being subjected to immunoblotting with anti-Akt, antipAkt (Ser473), anti-ERK, and anti-pERK antibodies. (B) Data quantification of ADP-induced ERK and Akt phosphorylation (****P<0.0001; ***P<0.001). Each experiment was repeated at least 3 times with blood collected from a group of 5-8 mice each time.

In summary, our data provides evidence that two week exposures to JUUL e-cigarettes vapes results in enhanced *in vitro* and *in vivo* platelet function, which was also confirmed by biochemical means.

Section 2.5. Discussion

The JUUL e-cigarette has been gaining popularity at an alarming rate since its introduction into US market in 2015, and by 2018 has overtaken more than half of the e-cigarette market (33, 35, 93). In this connection, it was marketed by JUUL labs, the manufacturer, as an e-cigarette device with a higher safety profile than traditional cigarettes (38); which we know is not supported by evidence. It has a unique USB- like design and comes with pre-filled pods that makes it appealing to use and easy to carry. Consequently, its use increased significantly among smokers and non-smokers, but also especially (and of more concern) among the youth (62, 83, 94). In term of the cardiovascular safety of e-cigarettes, we recently showed that exposure to traditional e-cigarette enhances physiological hemostasis and increases risk of thrombosis via modulating platelet function, in adult mice (75). However, whether the JUUL e-cigarette exerts negative health effects, namely increases the risk of thrombosis-dependent cardiovascular disorders remains to be investigated. Indeed, given the wide popularity and the unique features of JUUL e-cigarette, it is paramount to establish the cardiovascular safety of these devices. Thus, this issue was investigated by employing a mouse whole-body *in vivo* exposure model and a protocol that mimics real-life exposure scenarios of a daily JUUL user (69). It has been recently reported that daily JUUL users consume 10 pods per one month, which equals one pod every three days. Since each pod can generate 200 puffs, we choose to expose mice to 70 puffs per day to resemble human settings (69). Given that JUUL e-cigarette has only recently been introduced into the market, human studies related to its effect on the general health, and the cardiovascular system (CV) in specific are non-existent. Thus, mouse models can serve

as a readily available and practical tools to provide rather rapid answers to some of the safety questions, which may need months and/or years to establish in human studies. Furthermore, human studies are complicated by the variability of e-liquids and various flavors used, patterns of use, user's experience, and the concomitant use of other tobacco products, which makes it hard to draw a conclusion. Notably, given these challenges in human studies, the current mouse model gives flexibility in mimicking many aspects of human use, and the literature does support the fact that mouse studies do "map" very well to humans in the context of tobacco exposure (75, 80-82).

Initially, we validated our model by measuring the levels of cotinine, a key biomarker of nicotine exposure (95, 96), in urine samples of JUUL e-cigarette and clean air exposed mice. The cotinine levels increased significantly in JUUL e-cigarette exposed mice, whereas it was undetectable in the clean air exposed mice. Indeed, these high cotinine levels are not surprising given the fact that each JUUL pod (200 puffs) contains a high concentration of nicotine (5% by weight); which is equivalent to 1 pack of cigarettes (97, 98). Furthermore, the use of nicotine salt results in high amounts of nicotine being delivered to users as the salt masks the bitter taste of nicotine. Consistent with our finding, a recent study showed that youth are exposed to higher concentrations of nicotine when they use JUUL, compared to combustible cigarettes (99). Taken together, JUUL e-cigarette delivers a high amount of nicotine to users, which raises concerns regarding potential negative impact of nicotine, especially among youth users (98); which warrants investigation. Of note, while e-cigarettes have potential toxicants, such as nicotine, propylene glycol/glycerol, and flavors (100, 101), the JUUL pods contain nicotine salts, whose toxicity is virtually unknown.

As for the effects of JUUL on the genesis of thrombosis-dependent CVD, studies are non-existent. Our mouse model data demonstrated for the first time that exposure to JUUL (two weeks) increases the risk of thrombosis, as reflected by the prolonged occlusion time compared to control mice. Additionally, JUUL exposure altered physiological hemostasis as evidenced by the shortened the tail bleeding time. A similar prothrombotic phenotype was also observed in short term exposure to traditional e-cigarettes (75). In support of these findings, a recent meta-analysis concluded that e-cigarettes should not be marketed as “cardiovascularly” safe devices as more evidence needs to be collected (102). Collectively, our findings clearly indicate that JUUL e-cigarettes pose a negative cardiovascular (thrombotic) health impact contrary to the claimed safety by JUUL labs (38).

In light of the observed prothrombotic phenotype and since platelets are key players in thrombus formation (103, 104), we sought to investigate the impact/effects of JUUL e-cigarette on platelet function. Our data revealed that JUUL exposure enhances platelet function, including aggregation, secretion, integrin GPIIb/IIIa activation, phosphatidylserine (PS) exposure, compared to clean air. Moreover, the phosphorylation of Akt and ERK, which serve as biochemical markers of platelet activity, was enhanced in response to JUUL e-cigarette exposure. These data indicate that JUUL e-cigarette exposed platelets are hyperactive, which could explain- at least partially, the observed prothrombotic phenotype. Supportingly, our previously published data with traditional e-cigarette exposure showed similar trends of increased platelet function, in mice (75). These data are also consistent with a recent human study in which acute e-cigarette use was found to increase platelet activation (76).

Section 2.6. Conclusion

In conclusion, our study revealed for the first time that whole-body JUUL e-cigarette exposure alters physiological hemostasis and increases the risk of thrombosis, in mice; in part by modulating platelet function. Importantly, these findings provide clear/initial evidence that the JUUL e-cigarette is not safe as currently claimed, and should be the focus of further investigations. Additionally, our study should help increase awareness of the negative health consequences of these devices, especially among the youth, including those who had never smoked before but tried JUUL with the perception that these devices are safe. Given the current epidemic of e-cigarette use, specifically JUUL, we expect our data to help guide/inform the FDA in instituting and implementing stricter regulations to limit the use of JUUL devices.

CHAPTER III: A Novel Antibody Targeting The Second Extracellular Loop of The Serotonin 5-HT_{2A} Receptor Inhibits Platelet Function.

Section 3.1. Abstract

Background: Serotonin (5-hydroxytryptamine or 5-HT) is known to be a weak agonist involved in thrombus formation. 5-HT cannot induce platelet aggregation on its own. Yet, when the secreted 5-HT binds to its G-protein Coupled Receptor (GPCR; i.e. $5HT_{2A}R$), 5-HT acts to amplify platelet functional responses (e.g., aggregation). Thus, $5HT_{2A}R$ mediated responses are more involved in the secondary amplification of platelet aggregation in the growing thrombus. Therefore, 5-HT can be seen as a weak inducer of platelet activation, but it is an important amplifier of aggregation triggered by agonists such as ADP, collagen, and epinephrine, thereby enhancing thrombogenesis. $5HT_{2A}Rs$ are of clinical interest to the medical community as they are implicated in cardiovascular diseases. Efforts to develop antagonists for $5HT_{2A}R$ as therapy in cardiovascular diseases have thus far failed due to these reagents having deleterious side-effects and/or to lack of selectivity. **Methods:** In light of research efforts that identified that the $5HT_{2A}R$ ligand binding domain lies in the second extracellular loop (EL2; P^{209}-N^{233}), we developed an antibody, i.e. referred to as $5HT_{2A}RAb$ against the EL2 region Q^{216}-N^{233}, and characterized its pharmacological activity. Thus, we utilized C57BL/6J mice (10-12 weeks old) to analyze the inhibitory effects of the $5HT_{2A}RAb$ on platelet activation *in vitro, ex vivo,* and on thrombogenesis *in vivo*. **Results:** Our results indicate that the $5HT_{2A}RAb$ inhibits platelet aggregation *in vivo*, *ex vivo*, and that it prolongs the thrombus occlusion time, but it did not modulate the tail bleeding time, in mice. **Conclusion**: Our findings

document that $5HT_{2A}$RAb exhibits platelet function blocking-activity and protects against thrombogenesis without impairing normal hemostasis.

Section 3.2. Introduction

Platelets are indispensable to physiological hemostasis (105, 106). When a blood vessel is injured, platelets are able to interact with the unmasked/exposed subendothelial matrix, which thereby leads to platelet adherence and activation (107, 108). Consequently, a number of intraplatelet signaling pathways are triggered, which result in a rise in intracellular calcium levels; secretion of granule content (109, 110) (e.g., serotonin/5HT and ADP); as well as liberate arachidonic acid (AA) from the membrane phospholipids (109). While ADP in turn interacts with its $P2Y_1$ and $P2Y_{12}$ G-protein coupled receptors (GPCRs), AA is acted upon by cyclooxygenase (COX-1) to produce PGH_2, which is metabolized by thromboxane A_2 synthase into TXA_2 (111, 112). The latter binds to its own GPCR (113-115), and thereby activates platelets. Moreover, another important aspect of platelet signaling involves binding of the secreted serotonin (5-hydroxytriptamine or 5-HT) to its platelet $5HT_{2A}$R GPCR in order to amplify platelet functional responses (e.g., aggregation) (116). Thus, several positive feedback loops are initiated to intensify the extent of aggregation by means of enhancing platelet responsiveness as well as recruiting other platelets to the site of injury, in order to achieve formation of a stable clot (116).

Although platelet activation is a vital component of hemostasis, improper activation and aggregation may lead to thrombosis and thromboembolism (43-46). Consequently, anti-thrombotic drug development efforts have focused on the discovery of reagents/drugs that modulate activation of platelets by their agonists (117, 118). The serotonin $5HT_{2A}$R,

which couples to Gq (119, 120), recently has been under drug targeting scrutiny as of late (55). While 5HT itself is a weak stimulator of platelet activation, it does however potentiate aggregation triggered by separate agonists, thereby enhancing thrombogenesis (24, 25). Another attractive aspect of targeting $5HT_{2A}R$ for therapeutic purposes centers on the notion that its anti-thrombotic activity and bleeding time adverse events can be separated (which has been demonstrated previously albeit the mechanism remains unknown to date (53-55). To this end, a host of antagonists for $5HT_{2A}R$ were developed throughout the years and investigated for their pharmacological activity (53-55, 121-123). Unfortunately, none have been approved for clinical use due to significant toxicity, and/or lack of selectivity for $5HT_{2A}R$, amongst others. One discernable reason for failure of $5HT_{2A}R$ antagonism thus far seems evident, as these agents were designed empirically, without "regard" to the $5HT_{2A}R$ binding domains information. Therefore, it is critical to understand the structural biology and signaling of $5HT_{2A}R$. In this connection, mapping of the $5HT_{2A}R$ ligand binding domain revealed that the ligand binding domain resides in the second extracellular loop (EL2) of the receptor protein, namely (P^{209}-N^{233}) (124-126).

Based on these considerations, in the present study, we custom-made and characterized the antiplatelet activity of a potentially $5HT_{2A}R$-function-blocking antibody that targets its EL2 region (abbreviated as $5HT_{2A}RAb$). Our studies revealed that this $5HT_{2A}RAb$ indeed inhibited serotonin-enhanced ADP-induced human/mice platelet aggregation *in vitro*, and *ex vivo* in mice. Also, $5HT_{2A}RAb$ inhibited serotonin-enhanced ADP-stimulated platelet secretion, glycoprotein (GP) IIb/IIIa activation, and phosphatidylserine (PS) exposure. The $5HT_{2A}RAb$ was also found to displace binding of the radiolabeled $5HT_{2A}R$ antagonist [^{3}H]Ketanserin from its binding sites; this effect was reversed by preabsorption of

$5HT_{2A}$RAb with its cognate peptide (Q^{216}-N^{233}). Moreover, as for its *in vivo* activity, mice treated with $5HT_{2A}$RAb had a significantly prolonged occlusion time, but their bleeding time was no different from that of the controls.

Section 3.3. Materials and Methods:

Section 3.3.1 Materials and Reagents

Serotonin hydrochloride, and ADP were obtained from Sigma Aldrich (St. Louis, MO). Stir bars and other disposables were from Chrono-Log (Havertown, PA). FITC-conjugated Annexin V, anti–P-selectin, and PAC-1 antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA). The $5HT_{2A}$ receptor targeting polyclonal antibody, designated $5HT_{2A}$RAb, was custom made and antigen-affinity purified by a commercial vendor, Abbiotec Inc (San Diego, CA). Platelet count was determined using an automated hematology analyzer (Drew Scientific Dallas, TX).

Section 3.3.2. Animals

C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME). Mice were housed in groups of 1–4 at 24^0C, under 12/12 light/dark cycles, with access to water and food ad libitum. All experiments involving animals were perform in compliance with the institutional guidelines, and were approved by the Institutional Animal Care and Use Committee.

Section 3.3.3. Human and Murine Platelet Preparation

Blood was drawn from healthy volunteers who denied taking any medication for 1 week prior to collection, or from C57BL/6 mice (8–10 weeks old). Mice were anesthetized and blood was collected from the heart. Coagulation was inhibited by 3.8% w/v sodium citrate solution (1 part sodium citrate to 9 parts blood). Human or mouse platelet rich plasma (PRP) was obtained by centrifugation at room temperature. Platelets were counted with an automated hematology analyzer and their count adjusted to $7x10^7$ platelets/ml, prior to each experiment. Washed human platelets were prepared as described in Karim et al (21). PRP was isolated in the presence of 0.37 units/ml apyrase and 10 ng/ml PGI_2 by centrifugation at 237g for 10 min at 20 °C. PRP was centrifuged at 483g for 10 min, and pelleted platelets were resuspended in HEPES/Tyrode's buffer (20 mMHEPES/NaOH, pH 6.5, 128 mM NaCl, 2.8 mM KCl, 1 mM $MgCl_2$, 5 mM D-glucose, 12 mM $NaHCO_3$, 0.4 mM NaH_2PO_4) containing 1 mM EGTA, 0.37 units/ml apyrase, and 10 ng/ml PGI_2. Platelets were washed and resuspended in HEPES/Tyrode's buffer (pH 7.4) without EGTA, apyrase, or PGI_2. The final platelet counts were adjusted to $7x10^7$ platelets/ ml, unless otherwise indicated.)

Section 3.3.4. *In vitro* Platelet Aggregation

Human PRP was incubated with the $5HT_{2A}$RAb for 5 min prior to experiments, except in case of the controls. Platelets were then activated with submaximal concentration of ADP (1 μM), in the presence or absence of 15 μM serotonin. Control experiments were conducted using the ADP agonist alone (10 μM). Platelet aggregation was measured by

the turbidometric method using a model 700 aggregometer (Chrono-Log Corporation, Havertown, PA). Each experiment was repeated at least 3 times, with blood collected from three different human donors, or pooled together from at least eight mice each time.

Section 3.3.5. *Ex vivo* Platelet Aggregation

Mice were injected with the $5HT_{2A}$RAb (150 mM) or vehicle (deionized water) using the tail vein route once daily for 5 days; in an attempt to mimic chronic administration of these drugs in patients. Mice were sacrificed one hour post last injection, and their blood collected. Platelets (with counts adjusted as described before) were stimulated with 1 μM ADP in the presence or absence of 15 μM serotonin, and platelet aggregation was measured. Control experiments were conducted using the ADP agonist alone (10 μM). Each experiment was repeated at least 3 times, with blood pooled from at least eight mice each time.

Section 3.3.6. Flow Cytometric Analysis

Human platelets ($2x10^8$) were incubated in the presence or absence of $5HT_{2A}$RAb (150 nM) for 5 minutes. Platelets were stimulated with 1 μM ADP in the presence or absence of 15 μM serotonin. Control experiments were conducted using the ADP agonist alone (10 μM). The reactions were stopped by fixing the platelets with 2% formaldehyde for 30 min at room temperature. Finally, platelets were incubated with FITC-conjugated Annexin V, anti– P-selectin, or PAC-1 antibodies at room temperature for 30 min in the dark. Finally, the platelets were diluted 2.5-fold with HEPES/ Tyrode buffer (pH 7.4). The samples were transferred to FACStubes and fluorescent intensities were measured using

a BD Accuri C6 flow cytometer and analyzed using CFlow Plus (BD Biosciences, Franklin Lakes, NJ).

Section 3.3.7. *In vivo* Thrombosis Model

These studies were performed as described previously (127, 128). Briefly, mice 8–10 weeks old received tail vein injections of the $5HT_{2A}$RAb (150 nM) or vehicle once daily for 5 days, and were anesthetized with isoflurane. Then, the left carotid artery was exposed and cleaned, and baseline carotid artery blood flow was measured with Transonic micro-flowprobe (0.5 mm, Transonic Systems Inc., Ithaca, NY). After stabilization of blood flow, 7.5% ferric chloride ($FeCl_3$) was applied to a filter paper disc (1-mm diameter) that was immediately placed on top of the artery for 3 min. Blood flow was continuously monitored for 30 min, or until blood flow reached stable occlusion (zero blood flow for 2 min). Data was recorded and time to vessel occlusion was calculated as the difference in time between stable occlusion and removal of the filter paper (with $FeCl_3$). An occlusion time of 30 min was considered as the cut-off time for the purpose of statistical analysis.

Section 3.3.8. Tail Bleeding Time

Mice were tail vein injected with the $5HT_{2A}$RAb (150 nM) or vehicle once daily for 5 days, before their hemostasis response was examined using the tail transection technique (127, 128). Briefly, mice were anesthetized and placed on a 37 °C homeothermic blanket. The 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 cessation was measured. Bleeding time of 15 min was considered as the cut-off time for

the purpose of statistical analysis.

Section 3.3.9. Radioligand Binding Displacement

Resuspended platelets (1 x 10^9 platelets/ml) were prepared as previously described(115, 129), before they were incubated with 2 nM [^{3}H]ketanserin at room temperature for 10 min. Next, increasing concentrations of the displacing $5HT_{2A}$RAb (10 nM-1 μM) were added for an additional 45 minutes. Next, the [^{3}H]ketanserin bound platelets were captured by running through 0.45 micron Millipore filters over a vacuum suction unit. The filters were then washed once and counted for radioactivity in a Beckman LS 6000 liquid scintillation counter. To calculate the non-specific binding, the same concentration of radio ligand was competed against 1000-fold excess of unlabeled ketanserin.

Section 3.3.10. Statistical Analysis

All experiments were performed at least three times. Analysis of the data was performed using GraphPad PRISM 7.0 statistical software (San Diego, CA) and presented as mean ± SD. The Mann-Whitney test was used for the evaluation of differences in mean occlusion and bleeding times. Analysis was also conducted using t-test, and similar results were obtained. Significance was accepted at $P<0.05$ (two-tailed P value), unless stated otherwise.

Section 3.4. Results

Section 3.4.1. $5HT_{2A}$RAb Inhibits Serotonin-enhanced Human Platelet Aggregation

Our initial analysis showed that serotonin (15 μM) alone does not induce platelet aggregation in human PRP (Fig. 3.1.A). In contrast, we did observe platelet aggregation when stimulating with ADP (1 μM), albeit it was a “weak” response given the subthreshold dose we used. Concurrent addition/stimulation with serotonin (15 μM) and ADP (1 μM) on the other hand resulted in a significant platelet aggregation response (Fig. 3.1.B). These results demonstrate that serotonin by itself cannot induce platelet aggregation, but that it does have the capacity to enhance ADP-induced aggregation. Given that serotonin produces its effects via the $5HT_{2A}$R, we investigated if the $5HT_{2A}$RAb that targets its ligand-binding site would inhibit serotonin-enhanced ADP-induced platelet aggregation. The data showed that the $5HT_{2A}$RAb did inhibit serotonin-enhanced ADP-induced platelet aggregation, dose-dependently (100-150 nM; Fig. 3.1.B), when compared to the vehicle control. We next confirmed that $5HT_{2A}$RAb (100 nM) does not affect the platelet activity in the absence of serotonin, and our results revealed that it did not exert any effects on ADP(alone; 10 μM)-induced aggregation (Fig. 3.1.C). As one would predict, serotonin-induced platelet shape change was inhibited by the $5HT_{2A}$RAb (100 nM; (Fig. 3.1.A).

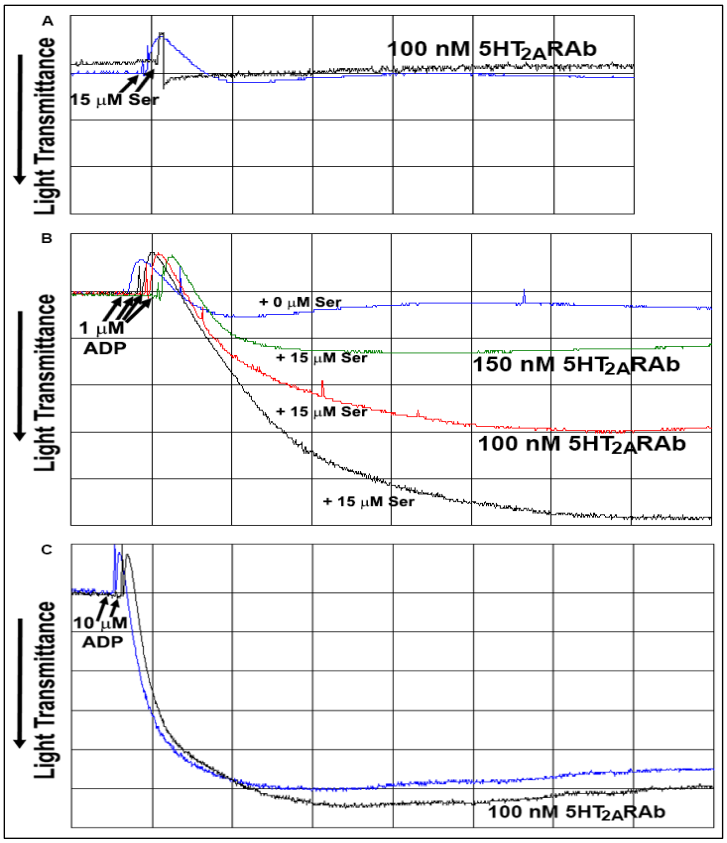

Figure 3.1. The $5HT_{2A}$RAb inhibits serotonin-enhanced ADP-induced human platelet aggregation *in vitro*. (A) Human PRP was activated with serotonin (15 μM), with or without pre-incubation for 5 min with $5HT_{2A}$RAb (100 nM). (B) Human PRP was preincubated with increasing doses of $5HT_{2A}$RAb (100–150 nM) for 5 min before being activated with a combination of ADP (1 μM) and serotonin (15 μM). (C) Human PRP was pre-incubated with $5HT_{2A}$RAb (100 nM) for 5 min before activation with ADP (10 mM). Each experiment was repeated at least 3 times, with blood obtained from three separate donors.

Section 3.4.2. 5HT$_{2A}$RAb Inhibits Serotonin-enhanced Mouse Platelet Aggregation *ex vivo*

We next examined investigated whether the aggregation "antagonistic" activity of the 5HT$_{2A}$RAb would persist if it was injected into live mice, under multi-dosing conditions. Thus, mice were intravenously injected once daily for 5 days with 5HT$_{2A}$RAb (100-150 nM). The results revealed that serotonin-induced platelet shape change was inhibited in the 5HT$_{2A}$RAb (150 nM)-treated mice (Fig. 3.2.A). Moreover, 5HT$_{2A}$RAb was found to exert inhibitory effects on serotonin-enhanced ADP-induced aggregation in platelets from 5HT$_{2A}$RAb injected mice, when compared to the vehicle control (Fig. 3.2.B). This finding demonstrates that the inhibitory effects of the 5HT$_{2A}$RAb are maintained under multi-dosing conditions in live animals.

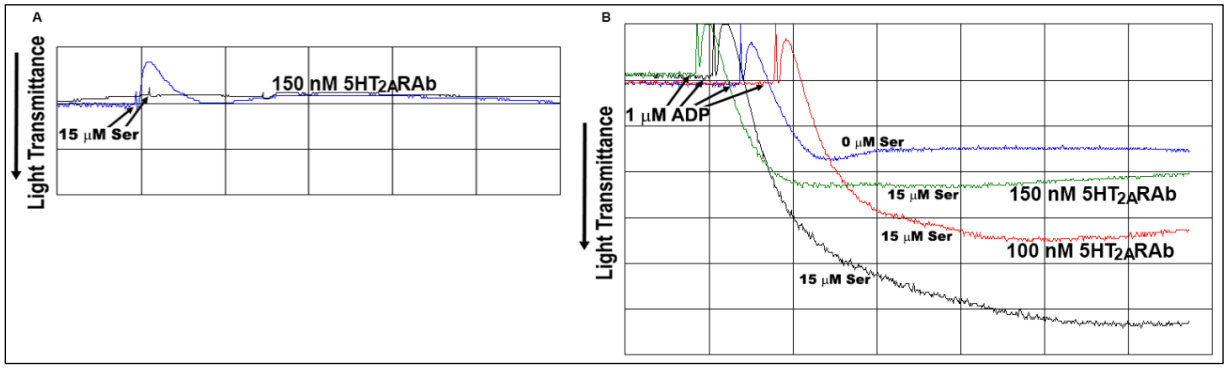

Figure 3.2. **The 5HT$_{2A}$RAb inhibits serotonin-enhanced ADP-induced mouse platelet aggregation *ex vivo*.** (A) Mouse PRP obtained from 5HT$_{2A}$RAb-injected (150 nM; once daily for 5 days) or control mice was stimulated with serotonin (15 μM). (B) Mouse PRP obtained from either vehicle-injected mice (once daily for 5 days) or 5HT$_{2A}$RAb-injected mice (100-150 nM; once daily for 5 days) was stimulated with submaximal concentration of ADP (1 μM) in the presence or absence of serotonin (15

μM). Each experiment was repeated at least 3 times, with blood pooled from at least eight mice each time.

Section 3.4.3. The $5HT_{2A}$RAb inhibits serotonin-enhanced ADP-induced platelet dense granule secretion in human and mouse platelets ex vivo.

Agonist-induced granules release plays a very important role in the amplification of initial platelet activation during primary hemostasis (88-90). Accordingly, we determined if the serotonin antibody exerts any impact on serotonin-enhanced dense granule secretion (ATP). In line with our aggregation data, we observed $5HT_{2A}$RAb inhibition of serotonin-enhanced ADP-induced ATP secretion in human platelets (Fig. 3.3.A). This inhibition was also observed under ex vivo experimental conditions (Fig. 3.3.B).

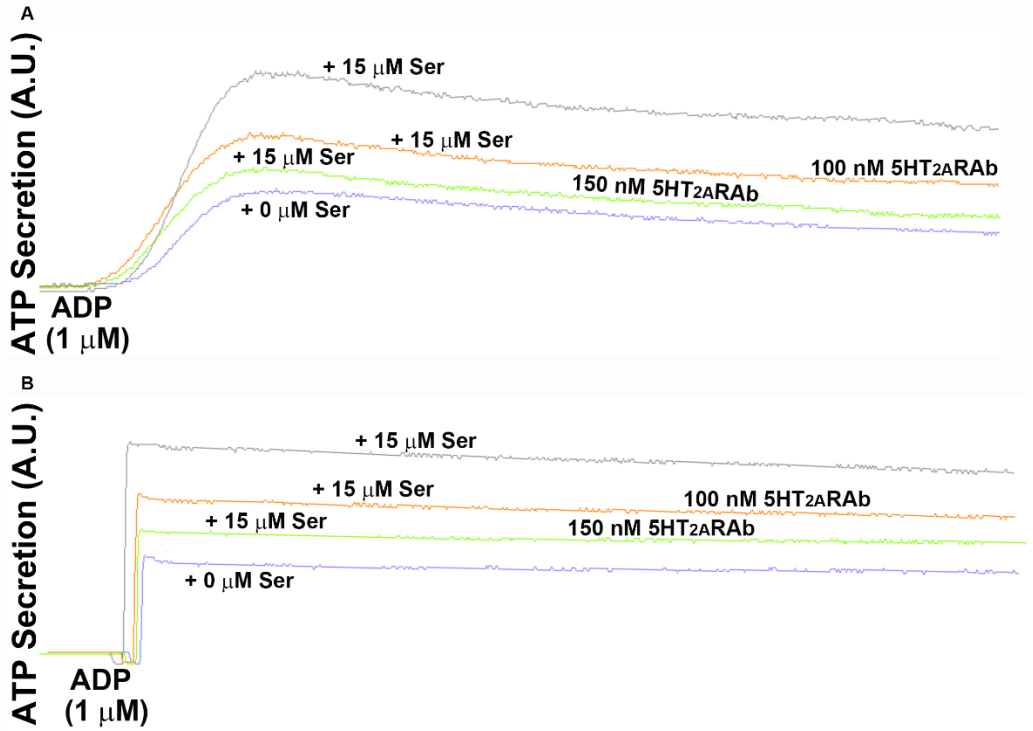

Figure 3.3. The $5HT_{2A}$RAb inhibits serotonin-enhanced ADP-induced secretion in human and mouse platelets ex vivo. (A) Human PRP was incubated with luciferase luciferin (12.5 µL) and increasing doses of $5HT_{2A}$RAb (100–150 nM) for 5 min before being activated with submaximal concentration of ADP (1 µM) in the presence or absence of serotonin (15 µM). (B) Mouse PRP obtained from $5HT_{2A}$RAb-injected mice (100-150 nM; once daily for 5 days) was preincubated with luciferase luciferin (12.5 µL) and stimulated with submaximal concentration of ADP (1 µM) in the presence or absence of serotonin (15 µM). ATP release (dense granules) was detected as luminescence and measured by a lumi aggregometer. Each experiment was repeated at least 3 times, with blood pooled from at least eight mice each time.

Section 3.4.4. $5HT_{2A}$RAb Inhibits Serotonin-enhanced Platelet α granule Secretion, Glycoprotein IIb/IIIa Activation and Phosphatidylserine Exposure

We next examined whether the $5HT_{2A}$RAb exhibits inhibitory effects on separate platelet functional responses that are also known to be enhanced by serotonin, namely α granule secretion (P-selectin expression), GPIIb/IIIa activation and phosphatidylserine (PS) exposure. Thus, human platelets treated with 150 nM $5HT_{2A}$RAb before being stimulated with ADP (1 µM) alone or with a combination of ADP (1 µM) and serotonin (15 µM). As expected, the $5HT_{2A}$RAb reversed the enhanced P-selectin expression, GPIIb-IIIa activation and PS exposure (Fig. 3.4.A-C), whereas it had no detectable effects on those responses induced by ADP alone. Based on these data, we conclude that $5HT_{2A}$RAb inhibits serotonin-enhanced ADP-induced expression of several markers of platelet activation, in addition to aggregation.

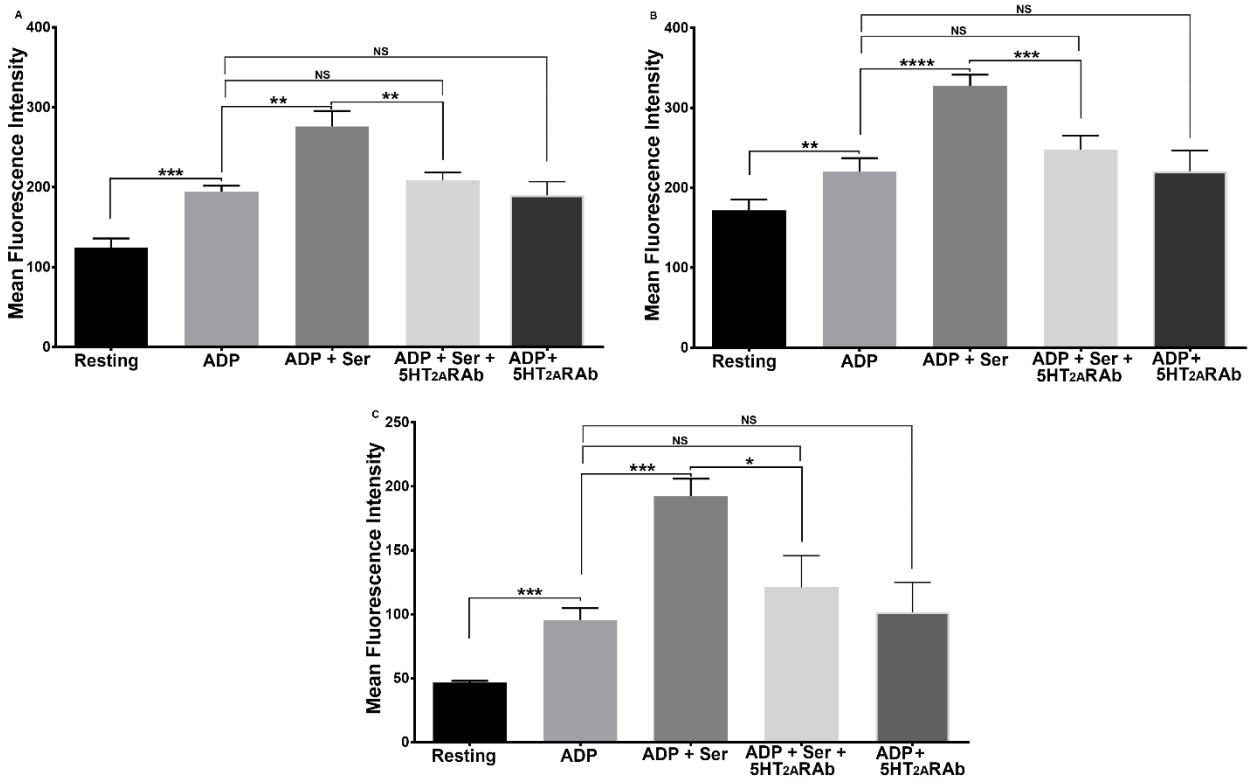

Figure 3.4. The $5HT_{2A}$RAb Inhibits Serotonin-enhanced ADP-induced α Granule Secretion, GPIIb/IIIa Activation and PS Exposure, in Human Platelets *in vitro*. Washed platelets were incubated in the presence or absence of the $5HT_{2A}$RAb (150 nM) for 5 minutes and then stimulated with ADP (1 μM), serotonin (15 μM) or ADP and serotonin (1 μM and 15 μM, respectively, for 3 minutes. The reactions were stopped by fixing the platelets with 2% formaldehyde for 30 min at room temperature. (A) Platelets were incubated with FITC-conjugated anti–P-selectin antibody, and the fluorescent intensity was measured by flow cytometry. (B) Platelets were incubated with FITC-conjugated PAC-1 antibody, and the fluorescent intensity was measured by flow cytometry. (C) Platelets were incubated with FITC-conjugated Annexin V antibody, and

the fluorescent intensity was measured by flow cytometry. Each experiment was repeated at least 3 times, with blood obtained from three separate donors.

Section 3.4.5. 5HT$_{2A}$RAb Prolongs the Thrombus Occlusion Time but not the Tail Bleeding Time

We next sought to assess the prospective of purposing the 5HT$_{2A}$RAb as an anti-thrombosis agent, *in vivo*. Thus, mice were tail-vein injected with 150 nM of 5HT$_{2A}$RAb before being subjected to the carotid artery $FeCl_3$ injury model experiment. Our results showed a significant increase in time to occlusion in the 5HT$_{2A}$RAb treated mice compared to the vehicle controls (Fig. 3.5.A). We next tested the effects of 5HT$_{2A}$RAb on hemostasis in order to assess bleeding as a potential adverse effect. We were pleasantly surprised that mice injected with 5HT$_{2A}$RAb had a bleeding time that was no different from the controls. (Fig. 3.5.B). Together, these results support the notion that 5HT$_{2A}$RAb protects/delays thrombus formation, and that it does so without increasing the risk of bleeding.

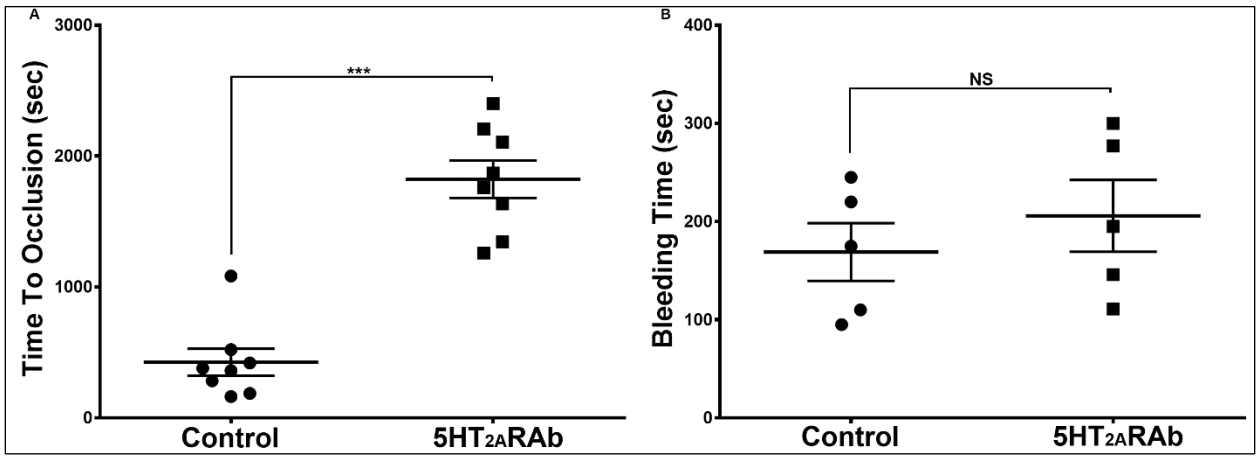

Figure 3.5. $5HT_{2A}$RAb prolongs the time to occlusion but not the tail bleeding time, in mice. Mice were injected via the tail vein with 150 nM $5HT_{2A}$RAb, once daily for 5 days before being subjected to the $FeCl_3$ thrombosis and tail bleeding time models. (A) Mean occlusion times for mice treated with 150 nM $5HT_{2A}$RAb or vehicle (n = 8). (B) Mean bleeding times for mice treated with 150 nM $5HT_{2A}$RAb or vehicle (n = 5). Each point represents the occlusion time or tail bleeding time of a single animal.

Section 3.4.6. $5HT_{2A}$RAb Displaces the $5HT_{2A}$R Antagonist Ketanserin from its Binding Sites

Finally, while the $5HT_{2A}$RAb exerts inhibitory effects on serotonin-enhanced platelet function, this is presumed to involve antagonism of the $5HT_{2A}$R. Indeed, increasing concentrations of the $5HT_{2A}$RAb (10 nM- 1 μM; inhibition of the Q^{216}-N^{233} amino acid region of the receptor's EL2 by $5HT_{2A}$RAb) did displace the radiolabeled $5HT_{2A}$R antagonist [^{3}H]ketanserin (2 nM) from its binding sites on human platelets (Fig. 3.6.). This effect was reversed by preabsorption of $5HT_{2A}$RAb with its cognate peptide (Fig. 3.6.). These data support the notion that $5HT_{2A}$RAb's platelet inhibitory effects involve interaction with the $5HT_{2A}$R ligand binding domain.

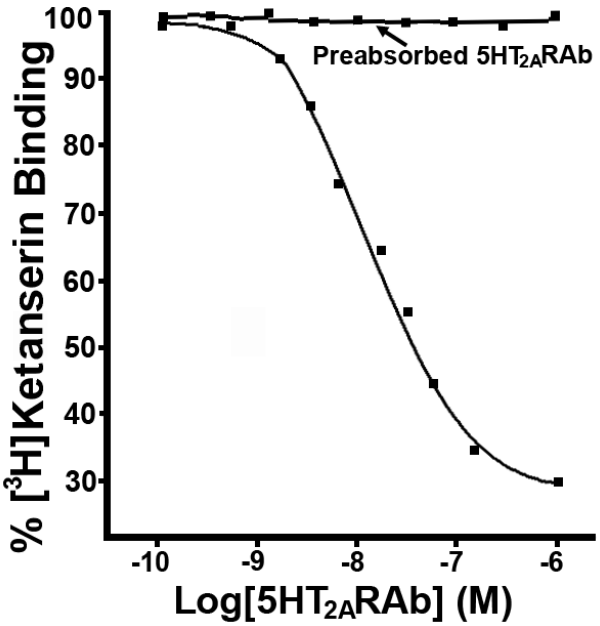

Figure 3.6. $5HT_{2A}$RAb displaces the $5HT_{2A}$R antagonist from its binding sites. Binding displacement of the radiolabeled $5HT_{2A}$R antagonist [^{3}H]ketanserin (2 nM) with increasing concentrations of $5HT_{2A}$RAb (10 nM-1 μM) in human platelets; preabsorption of $5HT_{2A}$RAb with its cognate peptide (i.e., Q^{216}-N^{233}) reversed its ability to displace binding of [^{3}H]ketanserin from the $5HT_{2A}$R.

Section 3.5. Discussion

According to the National Blood Clot Alliance, it is estimated that 274 people die every day from blood clots. This means one person dies every six minutes due to a blood clot (130-135). While the current FDA approved antiplatelet drugs reduce the risk of recurrent heart attacks, stroke, and death, they are associated with serious side effects (e.g., bleeding) which may outweigh their benefits (136-141). Thus, given the limitations of current thromboembolic therapy (e.g., resistance, and bleeding associated with plavix® and/or aspirin), developing safer drugs continues to be a sought after goal.

It has been demonstrated that serotonin/5HT amplifies aggregation triggered by separate agonists, thereby enhancing thrombogenesis (142, 143). This notion is supported by clinical data demonstrating that increased blood serotonin levels (144) and hyperactive 5-HT_{2A}Rs (144) correlate with cardiac events. Thus, efforts were undertaken to explore the potential beneficial effects of $5HT_{2A}$R antagonists as therapy in patients with cardiovascular disease. Unfortunately, while the serotonin $5HT_{2A}$ receptor ($5HT_{2A}$R) is known to play a critical role in platelet function *in vivo*, and has been implicated in several forms of cardiovascular disorders, currently there are no FDA-approved $5HT_{2A}$R antagonists available for managing thrombotic disease. Nonetheless, a host of such antagonists were designed and examined throughout the years (53-55, 121, 122). For example, the $5HT_{2A}$R antagonist ketanserin was shown in clinical studies (121) to reduce the incidence of myocardial infarctions in patients with coronary artery stenosis. However, these results were accompanied by deleterious side-effects due to lack of selectivity for $5HT_{2A}$R (121). Subsequent attempts to design antagonists with improved selectivity

profiles produced sarpogrelate, which (initially) showed promise in human clinical studies (122, 145). Unfortunately, this drug did not receive approval by the FDA due to limitations that are likely associated with its activity on $5HT_{2B}R$. Later, an experimental $5HT_{2A}R$ antagonist, namely AR246686 was synthesized and tested for its selectivity and efficacy (55). While AR246686 lacked affinity toward $5HT_{2B}R$ and $5HT_{2c}R$, and exhibited antithrombotic effects *in vitro* and *in vivo*, toxicity has prevented its clinical utility. It is to be noted that the empirical design of these agents and the lack of understanding the $5HT_{2A}R$ binding domains likely contributed to this failure. Consequently, it has been shown that $5HT_{2A}R$ ligand binding domain resides in the second extracellular loop (EL2; P^{209}-N^{233}) (124-126) which contains amino acid coordination (124, 125, 146-148) sites (S^{219}, L^{229}, A^{230}, and N^{233}), which are either shared or unshared between a number of ligands.

In our efforts to develop a $5HT_{2A}R$ antagonist, we custom made and characterized an antibody that targets its ligand binding pocket, i.e., the second extracellular loop Q^{216}-N^{233} sequence (designated $5HT_{2A}RAb$) for its function-blocking activity, in platelets. We demonstrated that this antibody has antiplatelet activities in both human and murine platelet models. Thus, we showed that the $5HT_{2A}RAb$ inhibited serotonin-enhanced agonist-induced platelet aggregation, whereas it exerted no effects on agonist(alone)-induced aggregation, not only *in vitro* but also *ex vivo* (under multi-dosing conditions). Moreover, the $5HT_{2A}RAb$ was also found to inhibit the ability of serotonin to enhance agonist-induced secretion, GPIIb/IIIa Activation and PS exposure. Furthermore, we found that the $5HT_{2A}RAb$ not only protected against thrombosis but that it did so without interfering with physiological hemostasis, in mice. Supportingly, it has been previously

indicated that targeting $5HT_{2A}R$ allows for the separation of anti-thrombotic activity and bleeding (53-55). Aside from findings that 5HT alone does not stimulate procoagulant responses (149), a likely explanation derives from the belief that the $5HT_{2A}R$ mediated responses are more involved in the secondary amplification of platelet aggregation in the growing thrombus, rather than playing a primary role in formation and stabilization of a hemostatic thrombus, like ADP and thromboxane A_2. Another explanation is that the antibody cannot penetrate the endothelial cell layer, and hence will not reach the smooth muscle cells; thereby this approach allows for the selective modulation of the platelet $5HT_{2A}R$, but not those of the smooth muscle, which are known to affect bleeding time. Together, these data support the notion that the $5HT_{2A}RAb$ would be superior to classical antagonists because its effects will be limited to the platelet $5HT_{2A}Rs$. It is noteworthy that an antibody against the GPIIb/IIIa, known as abciximab/ReoPro®, has previously been approved by the FDA for antithrombotic purposes. It is possible therefore that $5HT_{2A}RAb$ would also have clinical use. Notably, radioligand binding studies revealed that the aforementioned $5HT_{2A}RAb$ inhibitory effects derive from its ability to interact with the $5HT_{2A}R$ protein.

Section 3.6. Conclusion

In conclusion, our study constitutes the first investigation of function blocking antibody against $5HT_{2A}$ receptor function. Moreover, it provides novel information concerning a potential target site, i.e. EL2, for therapeutic intervention. Specifically, $5HT_{2A}RAb$ produces significant effects on platelet function and protects against thrombosis with no effect on bleeding. Collectively, these data suggest that targeting EL2 of $5HT_{2A}R$- for

which currently there are no interventions available for clinical use, may define a new class of anti-platelet drugs. Thus, future efforts will focus on generating a monoclonal version of the $5HT_{2A}$RAb, and a pharmaceutical formulation for clinical testing.

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Vita

Jean E. Montes started his college education at The University of Texas at El Paso (UTEP). Mr. Montes obtained a Bachelor's degree in Biochemical Engineering in the Fall of 2017. In the Spring of 2018, he was admitted into the Biomedical Engineering Master's program and joined Dr. Fatima Alshbool's laboratory in the Department of Pharmaceutical Sciences, School of Pharmacy at UTEP. Besides his graduate education, Mr. Montes also worked as a teaching assistant in the School of Pharmacy. During his career, he studied cardiovascular disease, specifically, that which is thrombosis-based via modulation of platelet function.

Mr. Montes' research projects focused on studying the health safety profile of JUUL, a popular electronic cigarette (e-cigarettes) in the U.S. Moreover, Mr. Montes' findings confirm that a novel antibody targeting the serotonin-receptor $5HT_{2A}R$ ligand binding domain could be used as an antithrombotic agent. Mr. Montes continues to expand his knowledge about cardiovascular diseases and thrombosis to improve health public awareness and help to develop novel antiplatelet agents for therapeutic use.

Mr. Montes will continue in the biomedical research field and will begin a doctorate degree in the spring of 2020.

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