CLINICAL STUDY PROTOCOL

The Thromboxane Receptor Antagonist to Block the Effects of Non-Platelet Thromboxane Generation and Improve Endothelial Function (TRAP) Trial

Protocol Number:	**H00017901**
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Investigational Drug:	**Ifetroban Oral Capsule**
IND Number:	**138088**
ClinicalTrials.gov Identifier:	**NCT03962855**
Study Sponsor:	**American Heart Association** **7272 Greenville Avenue** **Dallas, TX 75231**
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Approvals and Revision History

Approved By			
Name	**Title**	**Signature**	**Date**
Jeffrey J. Rade, MD	Professor of Medicine		October 31, 2020

Revision History	
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01	May 20, 2019 Initial Release
02	July 29, 2019 Amendment 1
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PROTOCOL SUMMARY

Title of Study	The Thromboxane Receptor Antagonism to Blunt the Effects of Non-Platelet Thromboxane Generation and Improve Endothelial Function (TRAP) Trial
Protocol Number	H00017901
Investigational Drug	Ifetroban Oral Capsules
Drug Manufacturer	Cumberland Pharmaceuticals Inc.
Objectives	The objectives of this study are: To determine if thromboxane receptor antagonism improves endothelial function in subjects with non-platelet thromboxane generation. To determine if thromboxane receptor antagonism reduces non-platelet thromboxane generation. To determine if thromboxane receptor antagonism reduces oxidative stress, inflammation and thrombotic potential. To determine if thromboxane receptor antagonism alters renal prostaglandin secretion and improves renal function.
Study Design	This is a phase 2b, single center, prospectively randomized, double-blinded, placebo-controlled clinical trial conducted at the UMass Medical School/UMass Memorial Medical Center. Subjects with cardiovascular disease on standard aspirin therapy with elevated non-platelet thromboxane generation defined by urine thromboxane B_2 metabolites >1145 pg/mg creatinine will be randomized to receive either ifetroban 250 mg capsules once daily or matching placebo.
Number of Subjects	57 subjects randomized 1:1 to ifetroban or placebo.
Treatment Duration	4 weeks
Study Endpoints	The primary trial endpoint will be the change from baseline in Reactive Hyperemia Index (RHI) measured by peripheral arterial tonometry (PAT).

	Secondary trial endpoints will be the change from baseline in on-treatment: 1) % flow-mediated vasodilation (FMD),and; 2) urine 11-dhTXB$_2$. Exploratory trial endpoints will be the change from baseline in on-treatment: 1) urine 8-iso-PFG$_{2\alpha}$; 2) plasma tissue factor; 3) plasma ICAM-1; 4) plasma activated protein C; 5) hs-CRP; 6) NT pro-BNP; 7) urine 2,3-dinor-6-ketoPGF$_{1\alpha}$ and ratio of 11-dhTXB$_2$ to 2,3-dinor-6-ketoPGF$_{1\alpha}$; 8) urine TXB$_2$, 6-ketoPGF$_{1\alpha}$ and the ratio of TXB$_2$ to 6-ketoPGF$_{1\alpha}$, and; 9) glomerular filtration rate.
Inclusion Criteria	Males and females 18-80 years of age with established cardiovascular disease who take ≥81 mg daily of aspirin and have thromboxane B$_2$ metabolites >1145 pg/mg creatinine on screening. Able to provide written consent and comply with protocol-specific procedures.
Exclusion Criteria	Chronic oral anticoagulation with non-vitamin K antagonist. Anticipated change or interruption in aspirin therapy during the study period. ST segment myocardial infarction within the past 30 days. Cardiac surgery within the past 30 days. Stage 4-5 renal failure or on renal replacement therapy. An ongoing uncontrolled severe inflammatory condition. Pregnant, intending to become pregnant or breast feeding. Known ifetroban or aspirin sensitivity Inability to perform vascular testing. Participation in another investigational drug trial within 30 days of randomization.
Safety Evaluation	Safety assessments will be performed at all study visits and will include physical examination and vital signs (blood pressure, pulse rate, respiration rate).

Statistical Methods	Descriptive statistics of patient characteristics will be generated using conventional methods. Continuous data will be compared between treatment groups using ANCOVA. Dichotomous data will be compared using chi-square analysis with a Yate's correction.

ABBREVIATIONS

Abbreviation	Definition
8-iso-$PGF_{2\alpha}$	isoprostane generated by free radical peroxidation of arachidonate
AA	arachidonic acid
ACS	acute coronary syndrome
ADP	adenosine diphosphate
AE	adverse event
AERD	aspirin exacerbated respiratory disease
ANCOVA	analysis of covariance
ASA	acetylsalicylic acid, aspirin
BMS	Bristol Myers Squibb
BUN	blood urea nitrogen
CABG	coronary artery bypass graft
CAD	coronary arterial disease
CEA	Clinical Events Adjudicator
CHARISMA	Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilization, Management and Avoidance
COX	cyclooxygenase one (prostaglandin-endoperoxide synthase or PTGS)
CPI	Cumberland Pharmaceuticals Inc.
CVD	cardiovascular disease
EC	endothelial cell
ECC	endocardial endothelial cell
ELISA	enzyme-linked immunosorbant assay
FDA	Food and Drug Administration
FHS	Framingham Heart Study
FMD	flow mediated dilation
FRIMS	Flexible Randomization and Inventory Management System
GFR	glomerular filtration rate
HOPE	Heart Outcomes Prevention Evaluation
HR	hazard ratio
hs-CRP	high sensitivity C-reactive protein
HUVEC	human umbilical vein endothelial cells
ICAM-1	intracellular adhesion molecule one
ID	identification
ife-AG	ifetroban acylglucuronide
IND	Investigational New Drug
INR	International Normalized Ratio
IPr	prostaglandin I_2 (prostacyclin) receptor
IRB	institutional review board
IsoP	isoprostane
IUPAC	International Union of Pure and Applied Chemistry

IV	intravenous
k_d	dissociation constant

Abbreviation	Definition
kg	kilogram
L	liter
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LnRHI	log transformed Reactive Hyperemia Index
LVEF	left ventricular ejection fraction
µg	microgram
µM	micromole
mg	milligram
MI	myocardial infarction
min	minute
mL	milliliter
mM	millimolar
N	number
nM	nanomolar
NO	nitric oxide
NT pro-BNP	N terminal pro-brain natriuretic protein
NYHA	New York Heart Association
PAH	pulmonary arterial hypertension
PCI	percutaneous coronary intervention
$PGF_{1\alpha}$	prostaglandin $F_{1\alpha}$
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGH_2	prostaglandin H_2
PGI_2	prostaglandin I_2, prostacyclin
PVD	peripheral vascular disease
REDCap	Research Electronic Data Capture
RHI	Reactive Hyperemia Index
RIGOR	Reduction in Graft Occlusion Rate study
ROS	reactive oxygen species
SAE	serious adverse event
SAR	serious adverse reaction
SMC	smooth muscle cell
STEMI	ST segment elevation myocardial infarction
t½	plasma terminal elimination half-life
TNF-α	tumor necrosis factor--α
TPr	thromboxane prostanoid receptor
TXA_2	thromboxane A_2
TXAS	thromboxane synthase
TXB_2	thromboxane B_2
TXB_2-M	thromboxane B_2-metbolites

1 Background

1.1 Overview of Thromboxane Biology

Thromboxane A_2 (TXA_2) is a signal-activated eicosanoid generated from the metabolism of arachidonic acid (AA) via the actions of the cyclooxygenase (COX) and downstream thromboxane synthase (TXAS) enzymes.[4] In healthy adults, the vast majority of TXA_2 is produced in platelets, where it not only mediates activation of the platelet in which it is formed, but is also released to locally amplify thrombotic stimuli by directly activating adjacent platelets and causing potent vasoconstriction.[5] Aspirin (ASA) exerts its antiplatelet effect by irreversibly inhibiting the COX-1 enzyme for the life of the platelet, thereby suppressing platelet TXA_2 generation and activation. In pathologic conditions such as cardiovascular disease (CVD), TXA_2 generation increases substantially in non-platelet tissues such as endothelial cell (EC) and in inflammatory cells (Figure 1). *Unlike platelet-derived TXA_2, standard doses of ASA are ineffective at suppressing non-platelet-derived TXA_2 due to the short circulating half-life of ASA ($t_{1/2}$ ~20 min), the ability of nucleated cells to regenerate COX-1 and formation of TXA_2 mediated by COX-2, which unlike COX-1 is not effectively inhibited by ASA*.

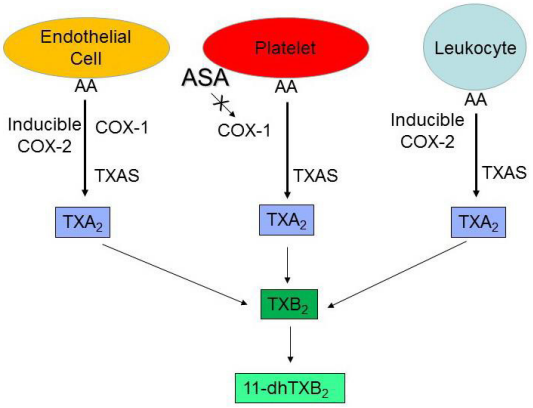

Figure 1. Sources of TXA_2 generation.

TXA_2 is a short-lived molecule ($t_{1/2}$ ~30 sec) that undergoes aqueous nucleophilic attack to form thromboxane B_2 (TXB_2), an inert eicosanoid that circulates in the bloodstream. TXB_2 itself is extensively metabolized via enzyme-mediated β-oxidation and dehydrogenation to dozens of inactive end-order metabolites (TXB_2-M) that are concentrated in the urine (Figure 1).[6] Measurement of one of these stable urine metabolites, 11-dehydroTXB_2 (11-dhTXB_2) was initially developed as a clinical indicator of ASA responsiveness based on data showing that ASA suppressed urine 11-dhTXB_2 to <400 pg/mg creatinine in >90% of healthy adults.[7] With the realization that non-platelet sources generate substantial amounts of TXA_2 in pathologic conditions, urine 11-dhTXB_2 is now considered a reflection of systemic non-renal TXA_2 generation. (TXA_2 generated by the renal glomeruli undergoes limited metabolism prior to secretion, thus urine TXB_2 is considered reflective of renal-specific TXA_2 generation.[8])

1.2 Thromboxane Receptor Biology

The biologic activity of TXA_2 is mediated through the binding to the thromboxane-prostanoid receptor (TPr), a G-protein coupled transmembrane protein present on a diverse array of cell types, including EC, vascular smooth muscle cells (SMC) and cardiac myocytes.[9-12] Two isoforms exist that can form heterodimers, TP_α, which is widely distributed, and TP_β, which is restricted to limited cell types such as EC.[13] High-affinity ligands for TPr are TXA_2 and PGH_2 (K_d 6.19-16 nM), but isoprostanes such as 8-iso$PGF_{2\alpha}$ that form non-enzymatically from arachidonic acid under conditions of oxidative stress, are also physiologically important ligands (K_d 31.8 nM).[14] Key cellular effects of TPr activation are shown in Table 1. In whole blood vessel preparations, TPr activation inhibits nitric oxide (NO) production and endothelial-dependent vasodilation.[15] Several

reports have shown that TPr activation not only increases superoxide generation but oxidative stress increases transport and stabilization of both TPr isoforms on cell membranes.[16-18] *In general, the TXA_2-TPr pathway's pro-coagulant, pro-inflammatory and vasoconstrictor effects counterbalance and in some cases directly antagonize the anti-coagulant, ant-inflammatory and vasodilating effects of prostacyclin (PGI_2) and NO.*

Table 1. Cellular effects of TPr activation.[9, 11, 15-26]

Cell Type	Physiologic Effect
Platelet	↑Shape change, ↑Aggregation
SMC	↑Contraction, ↑Hypertrophy, ↑Proliferation
Myocyte	↑Apoptosis, ↑Arrhythmia
EC	↓NO, ↑ROS, ↑Adhesion Molecules, ↑Tissue Factor
Leukocyte	↑Activation, ↑Adhesion Molecules, ↑Tissue Factor
Lymphocyte	↑Cytokine release, ↓Acquired Immunity

1.3 TXA_2 Generation and Clinical Outcome

Several studies have shown that elevated urine levels of TXA_2 metabolites in patients with CVD on standard ASA therapy is associated with an increased risk of atherothrombotic events and death.[3, 27-30] Sub-studies from the HOPE and CHARISMA trials found that in patients with/high risk for CVD on ASA, those with urine TXB_2-M in the highest quartile had a ~1.7-fold increased risk of death, myocardial infarction (MI) or stroke compared those in the lowest quartile.[27, 29] Early interpretation of these results was that ASA failed to adequately inhibit platelet COX-1 activity in a subset of patients with CVD, leading to persistent TXA_2 generation, increased platelet reactivity and atherothrombosis. *It is now understood that ASA is actually very effective at inhibiting platelet TXA_2 generation and that the observed persistent TXA_2 generation originates from non-platelet tissue that cannot be inhibited by standard ASA therapy.*

This concept was definitively demonstrated in the Reductions in Graft Occlusion Rates (RIGOR) study.[3] Given that ASA reduces the rate of vein graft (VG) thrombosis by 50% after coronary artery bypass graft (CABG) surgery[31], the RIGOR study hypothesized that failure of ASA to inhibit platelet activation would increase the prevalence of VG occlusion 6 months after CABG surgery. ASA responsiveness was assessed both by urine 11-dhTXB_2, which measures non-renal systemic TXA_2 generation, and by AA-induced platelet aggregation, which specifically assesses platelet TXA_2 generation. Similar to other studies[32-35], the RIGOR study found that ASA suppressed platelet TXA_2 generation in 95% and 99% of subjects 3 days and 6 months after CABG surgery, respectively. However, urine 11-dhTXB_2 was also elevated in 70% and 33% of subjects at these same time points, often to high levels (Figure 2A). While no association was found between the failure of ASA to suppress platelet TXA_2 generation and VG thrombosis, 11-dhTXB_2 ≥450 pg/mg creatinine measured 6 months after surgery was independently associated with VG occlusion (OR 2.6, P<0.02; Figure 2B) compared to values <450 pg/mg creatinine.[3] In an analysis of long-term outcome, 11-dhTXB_2 ≥450 pg/mg creatinine in 288 RIGOR subjects with suppressed platelet TXA_2 generation independently predicted 5-year MACE (adjusted HR 1.79, P=0.02) and death (adjusted HR 2.90, P =0.01) compared to lower values (Figure 3).[1] *These data provide compelling evidence that, in patients with CVD, unlike healthy individuals, there is substantial TXA_2*

generation from non-platelet sources that is not suppressed by standard ASA therapy and is a potent risk factor for atherothrombosis and death.

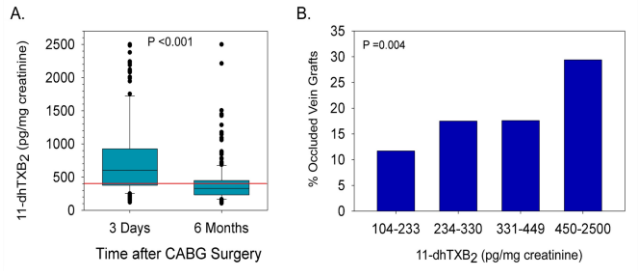

Figure 2. A. Urine 11-dhTXB$_2$ in RIGOR subjects 3 days and 6 months after CABG surgery.[3] Red line denotes 400 pg/mg creatinine. **B**. Percentage of occluded SVG 6 months after surgery in 229 subjects stratified by quartile of 11-dhTXB$_2$.[3]

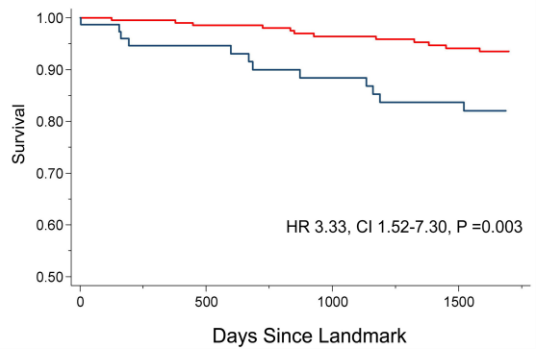

Figure 3. 5-year survival of 288 RIGOR subjects stratified by urine 11-dhTXB$_2$ <450 pg/mg creatinine (red line) or above (blue

To determine if TXA$_2$ generation predicts outcome in a large unselected population, TXB$_2$-M was recently measured 3042 subjects participating in Exam 8 Offspring and Exam 3 Omni cohorts of the Framingham Heart Study (FHS). The median urine TXB$_2$-M in the 1361subjects on ASA and the 1681 subjects not on ASA were 1145 and 4179 pg/mg creatinine, respectively. Regardless of ASA use, urine TXB$_2$-M above the respective median values was associated with more than a two-fold risk of long-term mortality (Figure 4; Rade, unpublished data).

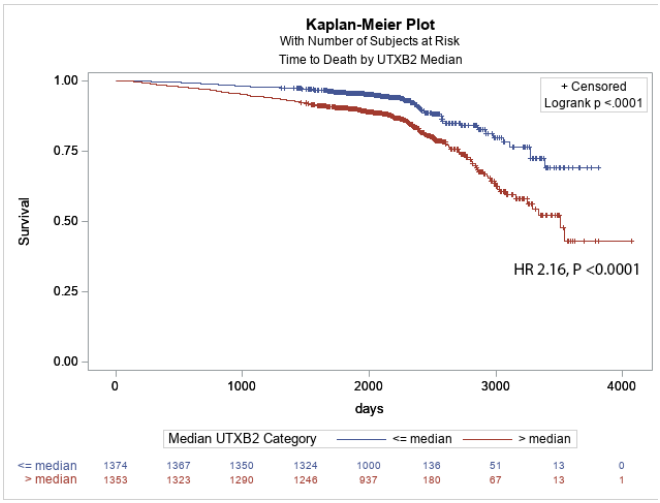

Figure 4. Long-term survival in 2727 FHS subjects stratified by mean urine TXB$_2$-M.

These data indicate that systemic TXA$_2$ generation is a potent risk factor for mortality, not only in patients with established CVD, but also in a general population and is independent of ASA use.

1.4 Factors Associated with Non-platelet TXA2 Generation

Multivariable modelling of RIGOR subjects with documented ASA-mediated suppression of platelet TXA$_2$ generation identified independent risk factors for non-platelet TXA$_2$ generation (Table 2).[2] Oxidative stress, as measured by urine 8-isoPGF$_{2\alpha}$, was identified as the strongest risk factor for non-platelet TXA$_2$ generation, accounting for nearly half of the modelled effect (Figure

5). In addition to being a marker for oxidative stress, 8-iso-$PGF_{2\alpha}$ is also a stable circulating eicosanoid capable of activating TPr to cause vasoconstriction and potentiate agonist-induced platelet activation.[36] Other independent risk factors are shown in Table 2.

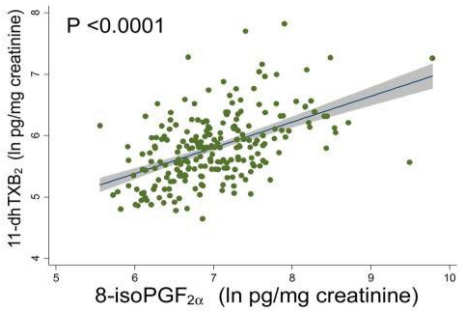

Figure 5. Linear regression of urine 11-dhTXB$_2$ to 8-isoPGF$_{2\alpha}$ in 228 subjects with suppressed platelet TXA$_2$ generation.[2]

Table 2. Independent risk factors for UTXB$_2$* identified by multivariable regression analysis.

	Standardized Coefficient	p-value	Dominance Weight
Urine 8-iso PGF$_{2\alpha}$ (ln pg/mg creatinine)	**0.442**	**<0.001**	**0.472**
Age (years)	0.239	<0.001	0.102
Female gender	0.129	0.015	0.093
White race (versus non-white)	-0.172	0.009	0.085
Statin therapy	-0.161	0.004	0.077
Creatinine (mg/dL)	-0.152	0.002	0.072
LVEF (%)	-0.113	0.032	0.048
ASA dose (81mg vs. >81 mg)	-0.145	0.004	0.052

*Normalized using natural log transform

1.5 Oxidative Stress Stimulates TXA$_2$ Generation in Endothelial Cells

While the predominant eicosanoid produced in healthy EC is PGI$_2$, TXA$_2$, is induced in dysfunctional EC where it counteracts the vasodilator effects of PGI$_2$ and nitric oxide (NO), thus impairing endothelial-dependent vasodilatation.[37, 38] Oxidative stress, a major mediator of EC dysfunction[39], stimulates TXA$_2$ and isoprostane generation in human EC (Figure 6A)[2]. Similar to porcine cerebral and retinal microvessels [40, 41], macrovascular EC generates substantial amounts of TXA$_2$ when directly stimulated with 8-iso-PGF$_{2\alpha}$ (Figure 6B), an effect that is inhibited by TPr blockade (Figure 6C). Furthermore, 8-isoPGF$_{2\alpha}$ may not be the most physiologic relevant isoprostane species to mediate this effect. For example, oxidative stress also results in formation of isothromboxanes, which would be expected to bind TPr more avidly than 8-isoPGF$_{2\alpha}$.[42] These data suggest that EC under oxidative stress are a potentially major source of non-platelet TXA$_2$ generation and that autocrine/paracrine stimulation of the TP receptor by TXA$_2$ and/or isoprostanes amplify this process. *Furthermore, they identify TP receptor blockade as a promising strategy to not only block the effects of TXA$_2$ but also inhibit its generation.*

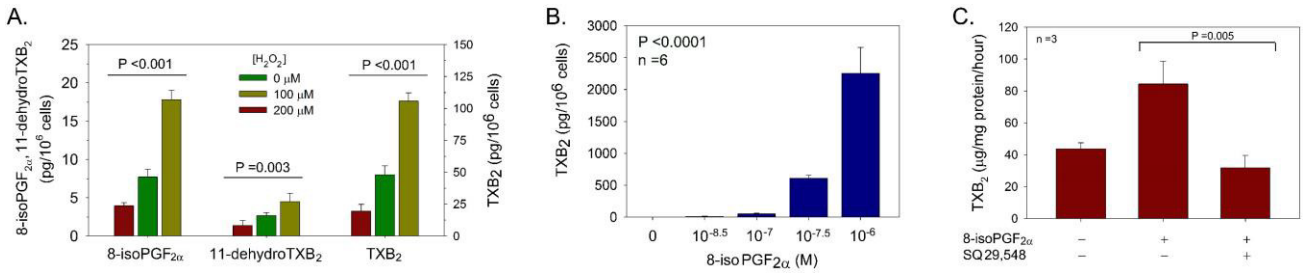

Figure 6. A. Oxidative stress induced by hydrogen peroxide (H_2O_2) increases generation of 8-isoPGF$_{2\alpha}$, 11-dhTXB$_2$ and TXB$_2$ measured by LC-MS/MS in the supernatants of human umbilical vein endothelial cells (HUVEC).[2] **B**. Direct exposure of HUVEC to 8-isoPGF$_{2\alpha}$ increases generation of TXB$_2$ measured by LC-MS/MS. **C**. TPr antagonism with SQ 29,548 (10^{-6}M) inhibits HUVEC TXB$_2$ generation in response to 8-isoPGF$_{2\alpha}$ (10^{-6}M) as measured by ELISA.

1.6 Non-platelet Thromboxane Generation and Atherothrombosis

Little is known about how non-platelet TXA$_2$ generation increases cardiovascular risk. While ASA-inhibited platelets could potentially still respond to non-platelet-derived TXA$_2$, in the RIGOR cohort 11-dhTXB$_2$ and 8-isoPGF$_{2\alpha}$ were independent of any tested parameter of platelet function[3] and in the CHARISMA trial the addition of clopidogrel did not reduce adverse events in subjects with elevated 11-dhTXB$_2$.[29] These data argue against non-platelet-derived TXA$_2$ or circulating 8-iso-PGF$_{2\alpha}$ mediating thrombosis in vivo by "priming" or potentiating platelet reactivity.

Stronger evidence exists for non-platelet TXA$_2$ and 8-iso-PGF$_{2\alpha}$ generation promoting atherothrombosis and impairing EC thromboresistance. In apo E-deficient mice, for example, atherogenesis is inhibited by the administration of a TP receptor antagonist but not by ASA.[43] Preliminary data reveal that TPr stimulation with isoprostane induces expression of multiple adhesion molecules in EC (Figure 7A; Rade unpublished data). TPr activation has been shown by others to potentiate cytokine-mediated TF expression in endothelial cells and monocytes[19, 44] and TF is also directly upregulated by oxidative stress (Figure 7B; Rade unpublished data). Additional evidence indicates that TPr inhibition blunts cytokine-mediated TXA2 generation (Figure 7C) and downregulation of thrombomodulin (TM), the key component of the protein C anticoagulant pathway that is critical for maintaining thromboresistance (Figure 7D; Rade unpublished data).[45-

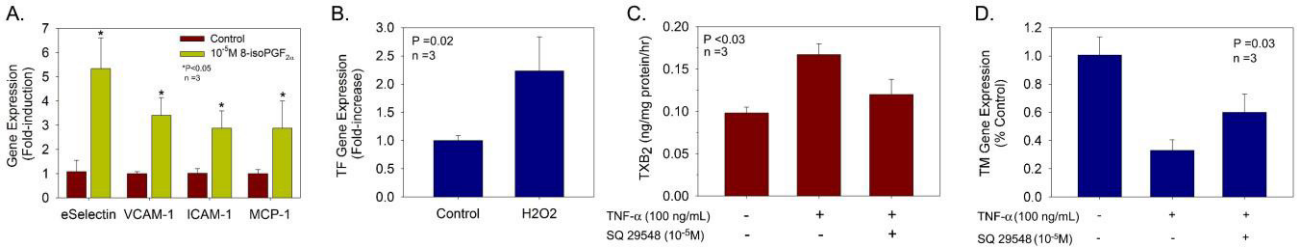

Figure 7. A. Isoprostanes stimulate adhesion molecule expression by HUVEC. **B**. Oxidative stress induced by 1-hour incubation with 200 µM H_2O_2 stimulates tissue factor expression in HUVEC. **C**. Cytokine-induced TXA2 generation in HUVEC is inhibited by TP receptor antagonism with SQ 29548. **D**. TP receptor antagonism with SQ 29548 blunts cytokine-mediated TM downregulation in HUVEC.

[48] These data suggest that autocrine/paracrine TPr activation by TXA_2 and/or isoprostane alter EC thromboresistance and provide the rationale using a TPr antagonist to reduce the risk of atherothrombosis.

1.7 Renal Effects of TXA_2 and TPr Inhibition

Prostaglandins play an important role in regulating renal blood flow and kidney function. The TPr is expressed in multiple places within the kidney (glomerulus, renal arterioles, thick ascending loop of Henle, collecting ducts and convoluted tubules) and renal TXA_2 generation is thought to play a role in regulating renal blood flow and glomerular filtration.[49, 50] TPr antagonism has been shown to inhibit renal vasoconstriction and prevent kidney injury in endotoxemic mice.[51] In diabetic apolipoprotein E-deficient mice, TPr antagonism was shown to attenuate oxidative stress and proteinuria.[52] In patients with cyclosporin-induced renal toxicity, infusion of a TXAS inhibitor reduced renal TXA_2 generation and improved glomerular filtration.[53] These data suggest TPr inhibition could potentially improve renal function in patients with CVD and increase non-platelet TXA_2 generation, especially those with heart failure.

1.8 Summary of Mechanistic Hypothesis Tested in this Study

Clinical and in vitro experimental data point to oxidative stress as a potent stimulus for endothelial TXA_2 generation. TXA_2 generation by vascular EC is hypothesized to be both a manifestation as well as a mediator of endothelial dysfunction. This provides the rationale for TPr antagonism as a means of improving vascular function. Figure 8 depicts a mechanistic model encompassing these concepts. Key features of the model include:

- Oxidative stress stimulates TXA_2 generation and inhibits PGI_2 generation by; 1) enhancing COX-1 activity and altering the relative activities of TXAS to prostacyclin synthase (PGIS), and; 2) non-enzymatic formation of isoprostanes.
- Autocrine/paracrine activation of the TPr by TXA_2 and/or isoprostanes; 1) potentiates TXA_2 generation via a positive feedback loop; 2) induction of adhesion molecules and other inflammatory mediators, and; 3) impairs thromboresistance by stimulating TF expression and inhibiting the TM-APC anticoagulant pathway.

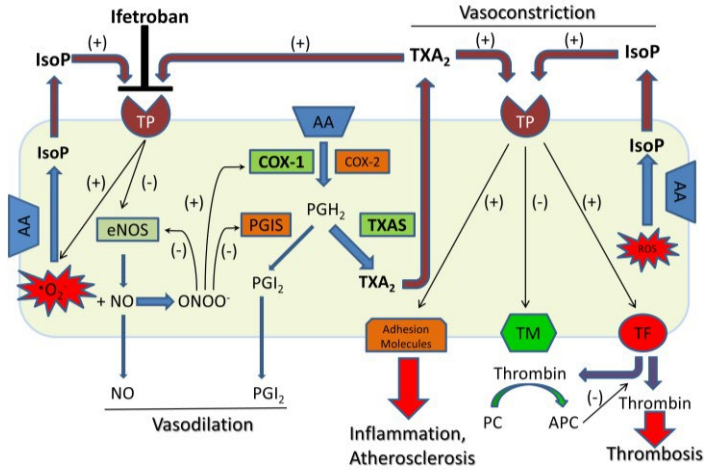

Figure 8. Proposed model for the effects of oxidative stress and mechanical stretch on EC/EEC eicosanoid generation and change in cellular phenotype. IsoP= isoprostane; ROS= reactive oxygen species; AA= arachidonic acid; TM= thrombomodulin; TF= tissue factor; APC= activated protein C.

2 Ifetroban.

2.1 Pharmacology

Ifetroban is a small potent and selective antagonist of the TPr with a molecular weight of 462.5 kD (Figure 9). Though readily absorbed after oral dosing, absorption is delayed after a meal and thus should be given to patients 30 minutes or more prior to a meal. Ifetroban has a plasma half-life of approximately 22 hours. Elimination is primarily by metabolism and biliary excretion.

Each capsule contains 250 mg of ifetroban. Other ingredients include mannitol, microcrystalline cellulose, crospovidone, magnesium oxide, colloidal silicon dioxide, and magnesium stearate. Capsules are filled into high density polyethylene bottles and sealed with screw-cap closures. Placebo for ifetroban capsules are formulated as a dry powder blend filled into capsules with similar matrix. Available data suggest the capsules will be stable for at least 48 months. A complete description of the pharmacology and toxicology of ifetroban is provided in the Investigator's Brochure.

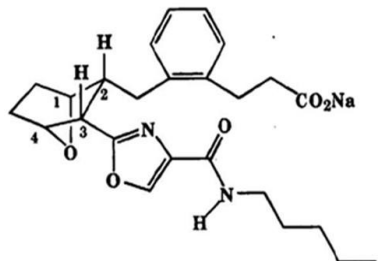

Figure 9. Structural formula of ifetroban.

2.2 Pre-Clinical and Human Studies

Ifetroban was initially developed for cardiovascular indications involving atherothrombosis and ischemia.[54] It has been the subject of extensive pre-clinical and clinical testing including eight randomized, placebo-controlled clinical trials in various cardiovascular indications, several of which included daily ifetroban oral doses of 250 mg for more than two weeks in subjects taking ASA. Ifetroban was well-tolerated in clinical studies with a cumulative enrollment of nearly 1,300 ifetroban-exposed subjects. Doses as high as 1000 mg were administered to healthy volunteers with no serious adverse events.

Ifetroban is currently under study for the treatment of several non-cardiac conditions including systemic sclerosis with and without pulmonary hypertension, ASA exacerbated respiratory disease, hepatorenal syndrome, portal hypertension and prevention of tumor metastases. By virtue of its direct TPr blocking effect and its potential ability to reduce TXA_2 generation by inhibiting a positive feedback loop involving the TPr, ifetroban is an ideal candidate to mitigate the effects of as well as potentially reduce non-platelet TXA_2 generation. A complete description of the pre-clinical and clinical experience with ifetroban, including extensive safety data is provided in the Investigator's Brochure.

2.3 Bleeding Risks

Ifetroban, by virtue of its ability to block the effects of TXA_2, has similar effects on platelet function and hemostasis as ASA, which exerts its effects by inhibiting TXA_2 generation. In subjects already on ASA therapy, the addition of ifetroban does not further impair platelet or hemostatic function and does not increase the risk of bleeding

TXA_2 is only one of several platelet agonists that play an important role in hemostasis. Platelet function studies reveal that ifetroban blocks platelet activation by AA and TXA_2 but not in response to ADP, thrombin or collagen. Thus, bleeding risk in subjects taking ifetroban plus dual antiplatelet

therapy (ASA + ADP receptor antagonist) would not be expected to be greater than in subjects taking dual antiplatelet therapy alone.

In a study of 17 subjects on stable warfarin therapy, the addition of 250 mg/d ifetroban for 7 days did not alter the INR (BMS Internal Report). The available clinical data has not demonstrated an increase in hemorrhagic events, though the number of subjects taking ifetroban and warfarin are limited (see Investigator's Brochure for details). The interaction of ifetroban and novel oral anticoagulants has not been studied.

2.4 Effect on Blood Pressure and Renal Function

TXA_2, through its activation of the TPr, is a potent vasoconstrictor. While ifetroban has been shown in pre-clinical animal models to reverse the hypertensive effects of infusions of arachidonic acid, it has not been shown to have significant hypotensive or adverse renal effects in humans: the incidence of hypotension and renal failure in all studies was <0.5% and not different with placebo treatment (see Investigator's Brochure for details).

2.5 Heart Failure

While ifetroban has not yet been studied specifically in a heart failure population, many of the subjects enrolled in the historic cardiovascular trials had recent myocardial infarctions and would have been expected to have had a diminished LVEF. In over 1500 subjects studied with cardiovascular disease, the incidence of new heart failure was low (≤1%) and no different between ifetroban and placebo treated subjects.

3 Study Objectives

This study will test the hypothesis that administration of the TPr receptor antagonist ifetroban will improve endothelial function by blocking the effects and generation of TXA_2 from non-platelet sources.

The objectives of this study are:

- To determine if thromboxane receptor antagonism improves endothelial function in subjects with non-platelet thromboxane generation.
- To determine if thromboxane receptor antagonism reduces non-platelet thromboxane generation.
- To determine if thromboxane receptor antagonism reduces oxidative stress, inflammation and thrombotic potential.
- To determine if thromboxane receptor antagonism alters renal prostaglandin secretion and improves renal function.

4 Study Design

The TRAP study is a phase 2b, single center, prospectively randomized, double-blinded, placebo-controlled outpatient clinical trial conducted at the UMass Medical School/UMass Memorial Medical Center. A schedule of study events is outlined in Appendix A.

4.1 Screening Phase

Patients with established cardiovascular disease on ≥81 mg daily ASA as part of their normal therapeutic regimen will be screened for non-platelet TXA_2 generation by measuring urine TXB_2 metabolites using either the AspirinWorks 11-dehydro Thromboxane B_2 Test Kit (Corgenix Inc., Bromfield, CO) or the essentially identical 11-dehydro Thromboxane B_2 ELISA Kit (Cayman Chemical, Ann Arbor, MI). Those with normalized values ≥1145 pg/mg creatine and who meet all other inclusion/exclusion criteria will be eligible for participation.

Urine TXB_2-M measured as part of a related on-going observational study of this same patient population under separate consent may be used to determine eligibility for this randomized trial.

4.2 Randomization

Eligible subjects will be randomized to receive either five 50 mg ifetroban oral capsules (250 mg total does) daily or matching placebo in a 1:1 ratio for 4 weeks. Double-blind randomization will occur at the first study visit using the UMass Flexible Randomization and Inventory Management System (FRIMS).

4.3 Study Interventional Phase

4.3.1 Study Visit 1 (Baseline Day 0, Outpatient)

- If not already done prior to this visit, subjects will be consented for the study.
- Compliance with uninterrupted daily aspirin use for the preceding 5 days will be verified.
- Baseline physical exam will be performed, vital signs will be recorded.
- Concomitant medications will be recorded.
- Baseline urine and blood samples will be obtained.
- Subjects will undergo baseline vascular testing.
- Subjects will be randomized.
- Subjects will be provided study drug enough for 35 days and instructed to take one pill daily. They will be instructed to continue all other medications, especially aspirin, as prescribed by their physician and to avoid unnecessary changes to their medical regimen.

4.3.1.1 Study Visit 2 (Interim Day 7 ± 3 Days, Phone Contact)

- Subjects will be contacted approximately 1 week after starting study drug to monitor for compliance and possible adverse events (AE).

4.3.1.2 Study Visit 3 (Follow-up Day 28 ± 3 Days, Outpatient)

- Subjects will take study drug on the day of the visit.

- Subjects will bring in unused study drug and pill counts will be performed.
- Physical exam will be performed, vital signs will be recorded.
- Concomitant medications will be recorded.
- Subjects will be assessed for possible AE.
- On-drug urine and blood samples will be obtained.
- On-drug physical exam with vital signs will be performed.
- On-drug vascular studies will be performed.

4.3.1.3 Study Visit 4 (Day 42 ± 3 Days, Phone Contact)

- Subjects will be contacted 2 weeks after stopping study drug to monitor for possible AE.

5 Study Population and Eligibility Criteria

5.1 Study Population

Patients suitable for this protocol are individuals with cardiovascular disease on standard aspirin therapy with elevated non-platelet thromboxane generation.

5.2 Inclusion Criteria

1. Males and females 18-80 years of age with established cardiovascular disease, defined by either:
    a. Angiographic (invasive or non-invasive) evidence of coronary atherosclerosis.
    b. Prior myocardial infarction.
    c. Positive stress test with documented peripheral or cerebrovascular disease.
2. Taking ≥81 mg daily ASA as part of their standard medical regimen.
3. Urine TXB_2-M >1145 pg/mg creatinine during screening.
4. Able to provide written consent and comply with protocol-specific procedures.

5.3 Exclusion Criteria

1. Chronic oral anticoagulation with a non-vitamin K antagonist.
2. Anticipated change or interruption in aspirin therapy during the study period.
3. STEMI within 30 days.
4. Cardiac surgery within 30 days.
5. Stage 4-5 kidney failure or on renal replacement therapy.
6. An uncontrolled ongoing severe inflammatory condition.
7. Pregnant, intending to become pregnant or breast feeding. (All women of child-bearing potential must have a negative pregnancy test within 24 hours of randomization.)
8. Known ifetroban or aspirin sensitivity.
9. Inability to perform vascular testing or comply with study protocol.
10. Participation in another investigational drug trial within 30 days of randomization.

6 Recruitment and Screening Procedures

6.1 Common Recruitment Procedures

Patients referred to the UMass Memorial Medical Center Heart and Vascular Interventional Laboratory for clinically-indicated cardiac catheterization who are participating in an ongoing observational clinical study (IRB #H00014594_3) will form the major screening population for this interventional trial. As part of this observational study, non-platelet thromboxane generation is assessed in each subject by measuring urine TXB_2-M. Subjects not participating in this screening study but who otherwise meet all the inclusion/exclusion criteria will be eligible to participate in this interventional trial.

6.2 Estimated Enrollment Period

This study will enroll a maximum of 57 subjects over a period of 30 months.

6.3 Informed Consent Procedures

Designated study personnel will explain to eligible subjects the purpose of the study, interventions, risk and benefits of participation and will answer questions. Wherever possible, subjects will be provided a copy of the UMass IRB approved consent form to review in advance of meeting with study personnel. All subjects who sign consent forms will be provide a full copy of the signed and executed document.

6.4 Confidentiality and HIPAA Requirements

All information collected on study participants will be stored in a confidential manner. Only approved study personnel will have access to data collected as part of the study. Consented study participants will be identified by a participant ID number on all study documents. Data will be stored securely using procedures described under Data Management.

6.5 Protection of Human Subjects

Protections for human subjects of research are required under Department of Health and Human Services regulations in 21 CFR parts 50, 56, and 312.

6.6 Summary of Risks and Benefits

6.6.1 Risks

6.6.1.1 Drug-related

Given its established safety profile, especially in patients with high-risk cardiovascular disease, the risks associated with ifetroban administration are expected to be minimal (see above section 2 and Investigator's Brochure). Given that both ifetroban and ASA inhibit TXA_2-mediate platelet activation, they have similar antiplatelet effects and bleeding risk.

Administration of ifetroban to subjects already taking ASA has not been shown to increase the risk of bleeding as the TXA_2 pathway in platelets is already maximally suppressed.

6.6.1.2 Blood draws

The risks of drawing blood include bleeding at the puncture site, bruising and pain. These would be expected to occur in a very small portion of the population.

6.6.1.3 Vascular Studies

The risks of vascular studies include discomfort in the arm during prolonged blood pressure cuff inflation.

7 Study Drug Handling

7.1 Drug Dispensing

50 mg ifetroban oral capsules and matching placebo will be provided by Cumberland Pharmaceuticals Inc. and packaged by the UMass Investigational Pharmacy into plastic bottles containing 175 pills. Bottle labels will contain instructions to take one pill daily in the morning, the name and contact information of the PI as well as a code that can be used in case of emergency unblinding.

7.2 Drug Storage, Accountability and Destruction

Study product will be stored prior to dispensing at the UMass Investigational Pharmacy at room temperature. Unused study drug will be returned by subjects at Visit 3 and disposed of by the UMass Investigational Pharmacy, which will hold all destruction documentation.

7.3 Emergency Unblinding

The UMass Investigational Pharmacy will maintain a log of study product codes that can be used in the unlikely event that emergency unblinding is required.

8 Outcome Determinations

8.1 Primary Endpoint

The primary trial endpoint will be change from baseline in on-treatment Reactive Hyperemia Index (RHI) measured by peripheral arterial tonometry (PAT) using the standard protocol described in Appendix C .

8.2 Secondary Endpoints

Secondary trial endpoints will be the change from baseline in on-treatment:

- Brachial % flow-mediated vasodilation (FMD) using the standard protocol described in Appendix B.

- Urine 11-dhTXB$_2$ as measured by mass spectrometry or immunoassay and normalized to urine creatinine.

8.3 Exploratory Endpoints

Exploratory trial endpoints will be the change from baseline in on-treatment:

- Urine 8-iso-PFG$_{2\alpha}$ as measured by immunoassay and normalized to urine creatinine.
- Plasma tissue factor as measured by immunoassay.
- Plasma ICAM-1 as measured by immunoassay.
- Plasma activated protein C as measured by immunoassay.
- hs-CRP as measured by immunoassay.
- NT pro-BNP as measured by immunoassay.
- Urine 2,3-dinor-6-ketoPGF$_{1\alpha}$ and ratio of 11-dhTXB$_2$ to 2,3-dinor-6-ketoPGF$_{1\alpha}$ as measured by immunoassay and normalized to urine creatinine.
- Urine TXB$_2$, 6-ketoPGF$_{1\alpha}$ and the ratio of TXB$_2$ to 6-ketoPGF$_{1\alpha}$ ketoPGF$_{1\alpha}$ as measured by immunoassay and normalized to urine creatinine.
- Glomerular filtration rate as calculated by the Cockroft-Gault formula.

9 Subject Safety

9.1 Institutional Review Board

The Study Protocol, Investigator Brochure, consent form, Investigational New Drug application (IND) and other relevant documents will be submitted to the UMass IRB for approval prior to commencement of the trial. Any amendment other than minor administrative changes, must be reviewed and approved by the IRB before implementation.

9.2 Food and Drug Administration

Use of ifetroban in this clinical trial is covered under an approved IND application to the US Food and Drug Administration (FDA).

9.3 Independent Clinical Event Adjudicator

An independent Clinical Event Adjudicator, with expertise in the field of cardiology will be identified and trained on the study protocol and Investigator Brochure prior to the first subject enrollment in this study. The role of this CEA is to provide an independent, expert review of data on clinical events based on protocol specific definitions outlined below.

9.4 Adverse Events

9.4.1 Definitions

9.4.1.1 Adverse Event

An adverse event (AE) is any untoward medical occurrence associated with the use of a drug in a participant, whether or not considered drug or biologic related. An AE can therefore be any undesirable sign, symptom or medical condition occurring after starting study drug, even if the event is not considered to be related to the pharmaceutical product. Study drug includes the drug under evaluation, and any reference or placebo drug given during any phase of the trial.

9.4.1.2 Suspected Adverse Reaction

A suspected adverse reaction (SAR) is any adverse event for which there is a reasonable possibility that the drug caused the event. "Reasonable possibility" suggests there is a causal relationship between the drug and the adverse event. "Suspected adverse reaction" implies a lesser degree of certainty about causality than adverse reaction, which means any adverse event caused by a drug.

9.4.1.3 Serious Adverse Events (SAE)

An adverse event or suspected adverse reaction is considered serious if the investigator or sponsor believes any of the following outcomes occurred:

- Death
- Life-threatening AE: Places the participant at immediate risk of death at the time of the event as it occurred. It does not include an AE that, had it occurred in a more severe form, might have caused death.
- Persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions.
- Inpatient hospitalization or prolongation of hospitalization.
- Results in congenital anomaly or birth defect.
- Important medical events that may not result in death, be life threatening, or require hospitalization may be considered a serious adverse event when, based upon appropriate medical judgment, they may jeopardize the participant and may require medical or surgical intervention to prevent one of the outcomes listed in this definition above. This determination is based on the opinion of either the investigator or sponsor (e.g., if either believes it is serious, it must be considered serious).

9.4.1.4 Laboratory Test Abnormalities

For laboratory test abnormalities that meet the definition of an SAE, that required the participant to have the investigational product discontinued or interrupted or required the participant to receive specific corrective therapy, the clinical diagnosis rather than the laboratory term will be used by the reporting investigator (e.g., anemia versus low hemoglobin value).

9.4.1.5 Assessment of Causal Relationship

A medically-qualified investigator must assess the relationship of any AE to the use of study drug, based on available information, using the following guidelines:

- **Not related:** There is not a reasonable causal relationship to the investigational product and the adverse event.
- **Unlikely related:** No temporal association or the cause of the event has been identified, or the drug or biologic cannot be implicated.
- **Possibly related:** There is reasonable evidence to suggest a causal relationship between the drug and adverse event.
- **Related:** There is evidence to suggest a causal relationship, and the influence of other factors is unlikely.

9.4.1.6 Assessment of Adverse Event Severity

The determination of adverse event severity rests on medical judgment of a medically-qualified Investigator. The severity of AEs will be graded using the following definitions:

- **Mild:** Awareness of sign, symptom, or event, but easily tolerated.
- **Moderate:** Discomfort enough to cause interference with usual activity and may warrant intervention.
- **Severe:** Incapacitating with inability to do usual activities or significantly affects clinical status and warrants intervention.

9.4.1.7 Expectedness

The expectedness of an AE or SAR shall be determined according to the most current Investigator's Brochure. Any AE that is not identified in nature, severity, or specificity in the current investigator's brochure is considered unexpected. Events that are mentioned in the Investigator's Brochure as occurring with a class of drugs or as anticipated from the pharmacological properties of the drug, but not specifically mentioned as occurring with the particular drug under investigation are considered unexpected.

9.4.2 Recording and Reporting of Adverse Events

The principal investigator, CEA and study team are responsible for monitoring the safety of participants enrolled into the trial. Events significant enough to necessitate discontinuation of study drug dosing will be captured on the AE electronic case report forms (eCRF).

All AEs/SAEs, except for those anticipated AEs, occurring from signed informed consent to 14 (+/- 3 days) after visit 3 will be captured on the AE/SAE eCRF. Unless exempted, all SAEs, whether or not deemed drug-related or expected, must be reported by the investigator or qualified designee within 24 hours of first becoming aware of the event. For this study, all deaths will be reported on the SAE eCRF, as well as the Death eCRF page. The investigator or qualified designee will enter the required information regarding the AE/SAE into the appropriate module of the eCRF, which will be shared with the CEA.

When additional relevant information becomes available, the Investigator will record follow-up information according to the same process used for reporting the initial event as described above. The Investigator will follow all reportable events until resolution, stabilization or the event is otherwise explained. The investigators are also responsible for promptly reporting SAEs to the IRB per our institutional policy..

9.4.3 Suspected Unexpected Serious Adverse Reaction

AEs that meet the criteria of serious, related to study drug, and unexpected per the Investigator's Brochure, qualify for expedited reporting to the regulatory authorities. The PI will notify the FDA and all participating investigators in a written IND safety report of an SAR that is serious and is unexpected, based on the opinion of the Adjudicator as soon as possible, but not later than 15 calendar days after the event is confirmed to be a serious, unexpected SAR and qualifies for expedited reporting.

10 Statistical Considerations

10.1 Sample Size Calculations

10.1.1 Primary Endpoint

Data from several studies was used to determine the power to detect a difference in RHI with drug treatment. In one study of PAT reproducibility in cardiovascular patients, the intra-patient standard deviation was 0.31.[55] In a meta-analysis of >1500 subjects with cardiovascular disease the mean RHI was 1.52.[56] Based on these data, a sample size of 48 total subjects (24 randomized each to ifetroban or placebo) is required to detect a 20% increase in RHI with over 85% power with a two-sided alpha of =0.05. Adjusting for 10% loss to follow up, we will need 52 randomized subjects.

10.1.2 Secondary Endpoints

Data from a preliminary study conducted at UMass (Keaney, unpublished data) in which FMD was measured in patients after non-STEMI ACS was used for primary endpoint power calculations. In that pilot study, mean FMD was 9.6 ± 2.0% in normal controls (n=16) and 4.6 ± 2.8% in ACS patients (n=22). Based on these data, a sample size of 48 total subjects (24 each randomized to ifetroban or placebo) is required to detect a 50% increase in FMD with 80% power for an analysis of covariance of each treatment separately with a two-sided alpha=0.05. Data from the RIGOR study was used to determine the power to detect a difference in non-platelet TXA_2 generation with drug treatment.[3] With a median 11-dhTXB_2 of 330 pg/mg creatinine, a 33% reduction would be to 220 pg/mg creatinine (reduction from 5.8 to 5.4 in the natural log scale) with a s.d. of 0.55 in the log scale. A sample size of 48 total subjects (24 each randomized to ifetroban or placebo) will have the ability to detect treatment group differences described above with 80% power for an analysis of covariance of each treatment separately with a two-sided alpha=0.05, assuming an $R^2 = 0.50$ with the baseline level and treatment included in the model.

10.2 Statistical Analysis Plan

Descriptive statistics of patient characteristics will be generated using conventional methods. The main analysis of the outcome data will be a standard ANCOVA approach (SAS PROC GLM). The full model will be fit for change in RHI(or other measure of interest) as the outcome for patient *i* based on treatment group (as two dummy (0,1) variables:x_1, x_2), a vector of patient characteristics $x_{3,I}$, and an error term e_i: $\ln(11\text{-dhTXB}_2)i=b_0 + b_1x_{1i} + b_2x_{2i} + b_3x_{3i} + e_i$. We will test the main effects (treatment as well as patient characteristics) included in the model with the treatment main effects always included. We will investigate interactions between patient characteristics and treatments to provide insight into the effect of these treatments on the outcomes overall and the effect in larger patient subgroups.

11 Data Management

Study data will be recorded in a Research Electronic Data Capture (REDCap) database in the secure regulated environment at the UMass Medical School. REDCap is a secure, web-based application designed to support data capture for research studies, providing 1) formatted data entry screens; 2) audit trails; 3) automated export procedures for seamless data downloads to statistical packages; 4) procedures for importing data from external sources; and 5) an Application Programming Interface that enables it to communicate with our web-based randomization system. Data will be exported from REDCap into SAS (SAS Institute) for all analysis.

12 Regulatory Issues

12.1 Ethics and Good Clinical Practice

This study will be carried out in compliance with the protocol. These procedures are designed to ensure adherence to Good Clinical Practice, as described in: 1) ICH Harmonized Tripartite Guidelines for Good Clinical Practice 1996, and; 2) US 21 Code of Federal Regulations dealing with clinical studies (including parts 50 and 56 concerning informed consent and IRB regulations). All investigators and study personnel agree to adhere to the instructions and procedures described in it and hereby to adhere to the principles of Good Clinical Practice to which it conforms.

12.2 Institutional Review Board

Before implementing this study, all required documents including the protocol, proposed informed consent form and Investigator's Brochure must be reviewed and approved by the UMMS IRB. Any amendments to the protocol, other than administrative ones, must be approved by this committee.

12.3 Informed Consent

The investigator or designee must explain to each participant the nature of the study, its purpose, the procedures involved, the expected duration, the potential risks and benefits involved and any discomfort it may entail. Each participant must be informed that participation in the study is voluntary and that he/she may withdraw from the study at any time and that withdrawal of consent will not affect his/her subsequent medical treatment or relationship with the treating physician.

The informed consent form(s)and any amendments must be submitted by the investigator for IRB approval.

12.4 Quality Control and Assurance

All CRFs and corresponding office and clinical laboratory records for each subject will be periodically independently audited by personnel from the UMass Office of Clinical Research. These audits will help to verify the accuracy and completeness of CRFs, to resolve any inconsistencies in the study records, and to assure that all protocol requirements, applicable FDA regulations, other requirements, and investigator's obligations are being fulfilled.

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14 Appendix A: Table of Study Events

Event	**Screening**	**Visit 1** Day 0	**Visit 2** Day 7±3	**Visit 3** Day 28±3	**Visit 4** Day 42±3
Informed Consent	X				
Medical History	X				
Physical Exam		X		X	
Vital Signs		X		X	
Concomitant Medications	X	X		X	
Study Drug Pill Counts		X		X	
AE Assessment		X	X	X	X
Vascular Studies					
FMD		X		X[a]	
PAT		X		X[a]	
Laboratory Studies					
UrineTXB_2-M	X				
Urine Prostanoids (8-iso-$PGF_{2\alpha}$; 6-keto$PGF_{1\alpha}$; 2,3-dinor-6-keto$PGF_{1\alpha}$)		X		X	
Urine Creatinine	X	X		X	
Plasma ICAM-1; APC; TF		X		X	
Serum creatinine; hs-CRP; NT pro-BNP		X		X	

[a]Performed while still on study drug.

15 Appendix B: Brachial Reactivity

15.1 Introduction

Healthy blood vessels dilate in response to an increase in blood flow, a phenomenon known as flow-mediated dilation (FMD).[57] FMD is predominantly a function of endothelial nitric oxide (NO) release that relaxes underlying vascular smooth muscle cells. Endothelial release of PGI_2 also plays a role in mediating FMD. TXA_2, in addition to being a potent platelet activator, is also a potent vasoconstrictor that antagonizes the effects of NO and PGI_2.

Vascular dysfunction is associated with the development and presence of atherosclerosis and characterized by an impaired vasodilatory response to blood flow. FMD is traditionally measured non-invasively by ultrasound in the brachial artery following a period of blood flow cessation caused by an inflated blood pressure cuff.[58] Typically, the cuff is inflated to at least 50 mm Hg above systolic pressure to occlude arterial inflow for 5 minutes. This causes ischemia and consequent dilation of downstream resistance vessels via autoregulatory mechanisms. Subsequent cuff deflation induces a brief high-flow state through the brachial artery (reactive hyperemia) to accommodate the dilated resistance vessels. The resulting increase in shear stress causes the brachial artery to dilate. The ratio of the brachial artery diameter after the occlusive period to that at baseline is recorded as the percent FMD. Impaired FMD is associated with cardiovascular risk factors and the development of clinical cardiovascular disease. The protocol that will be used is based on guidelines published by the American College of Cardiology.[59]

15.2 Protocol

15.2.1 Subject preparation

1. Prior to the study, ensure the patient has fasted for at least 4 hours, and has refrained for at least 8 hours from caffeine, tobacco, vitamins or medications that might affect vascular tone. The patient may wish to use the restroom prior to the study.
2. The brachial reactivity study will be conducted in a quiet, dimly lit, temperature-controlled exam room to reduce fluctuations in vascular tone.
3. Cell phones or paging devices should be silenced, and restrictive clothing that could interfere with blood flow to the arms should be removed. The patient should also remove watches, rings, or other jewelry on the hands or fingers.
4. The patient should be supine and comfortable for 15 minutes so as to attain a cardiovascular steady-state. Place the two arm supporters along each of the patient's sides.
5. Place a blood pressure cuff on the arm to be occluded during the brachial reactivity study. Apply the cuff snugly, but without excess pressure. Do not inflate the cuff at this time.
6. The brachial artery is imaged above the antecubital fossa in the longitudinal plane (Fig. 1) using a linear array transducer with a minimum frequency of 7 MHz, attached to a high-quality mainframe ultrasound system.

7. A segment with clear anterior and posterior intimal interfaces between the lumen and vessel wall is selected for continuous 2D grayscale imaging. During image acquisition, anatomic landmarks such as veins and fascial planes are noted to help maintain the same image of the artery throughout the study. A stereotactic probe-holding device can be helpful.

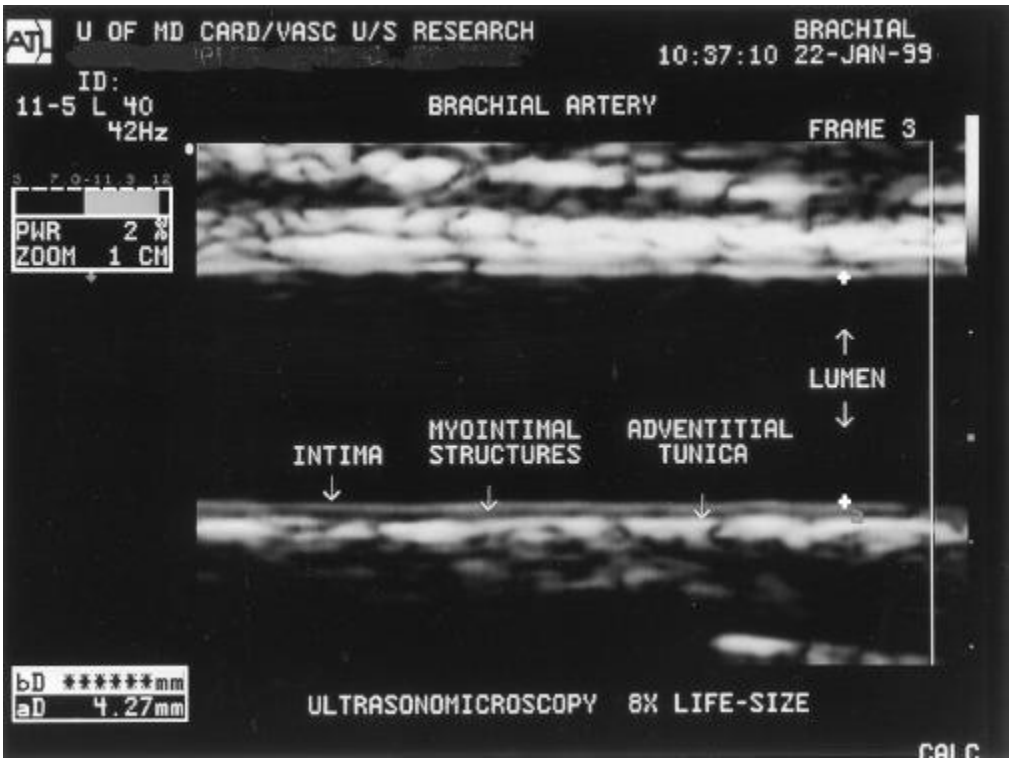


Figure 1. Ultrasound image of the brachial artery (longitudinally) at 8× magnification, 11-MHz transducer frequency annotated for anatomic landmarks.

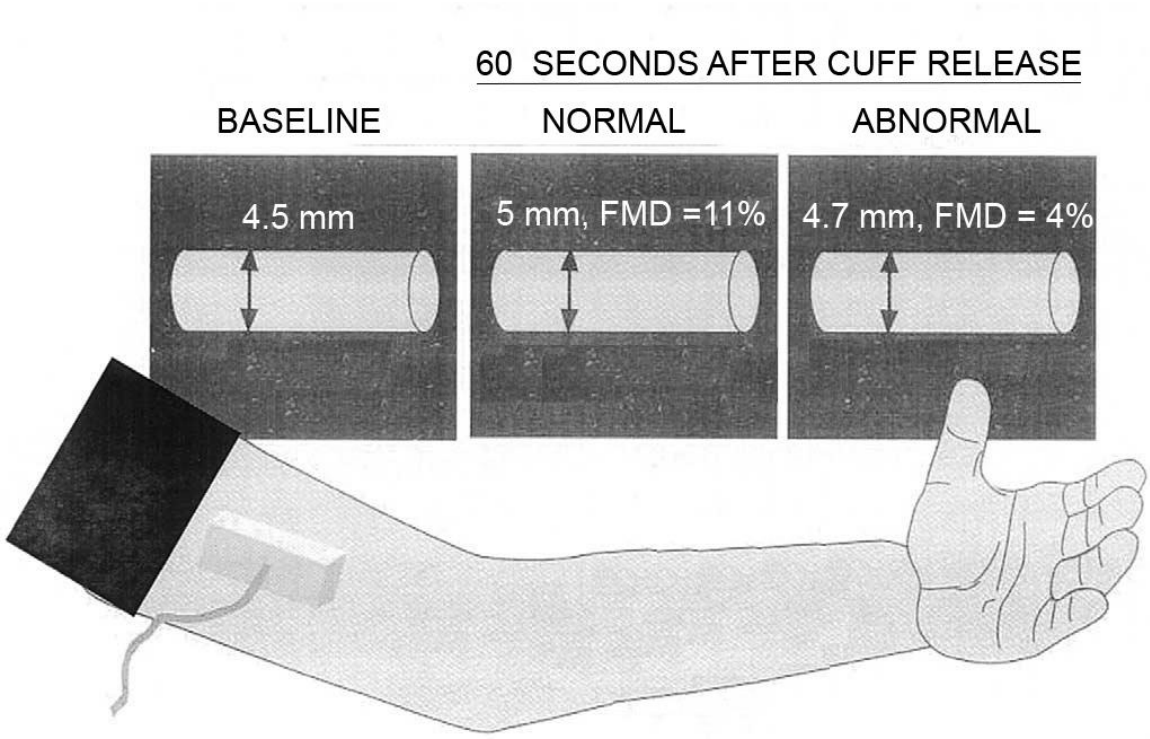
15.2.2 Conduct of the Brachial Reactivity Study

1. Tell the patient that you are going to inflate the cuff for the occlusion phase and that he or she should stay relaxed and not move the fingers.

2. Rapidly inflate the blood pressure cuff to a supra-systolic pressure of 60mmHg above the patient's systolic pressure or 200mmHg, whichever is higher. A mid-artery pulsed Doppler signal is obtained to confirm cessation of blood flow.

3. After 5 minutes, rapidly deflate the blood pressure cuff. A midartery pulsed Doppler signal is obtained upon immediate cuff release and no later than 15 s after cuff deflation to assess hyperemic velocity.

15.3 Representative Results



4.

Figure 2. Schematic drawing of ultrasound imaging of the brachial artery and representative normal and abnormal results.

16 Appendix C: Peripheral Arterial Tonometry

16.1 Introduction

Assessment of digital vasodilator responses using a commercially-available fingertip peripheral arterial tonometry (PAT) device (EndoPAT; Itamar Medical LTD, Caesarea, Israel) is a novel measure of vascular function that provides different but complimentary information compared with FMD.[60]With this device, a beat-to-beat plethysmographic record of the finger arterial pulse wave amplitude is captured using pneumatic probes placed on the index finger of each hand (Figure 1). The same reactive hyperemia technique that is used to measure FMD is used for Endo-PAT and the two techniques can be performed simultaneously. The baseline digital pulse amplitude is a measure of local arterial tone in the fingertip. The PAT ratio records post-ischemic arterial responsiveness to reactive hyperemia produced by a cuff occlusion in the forearm.

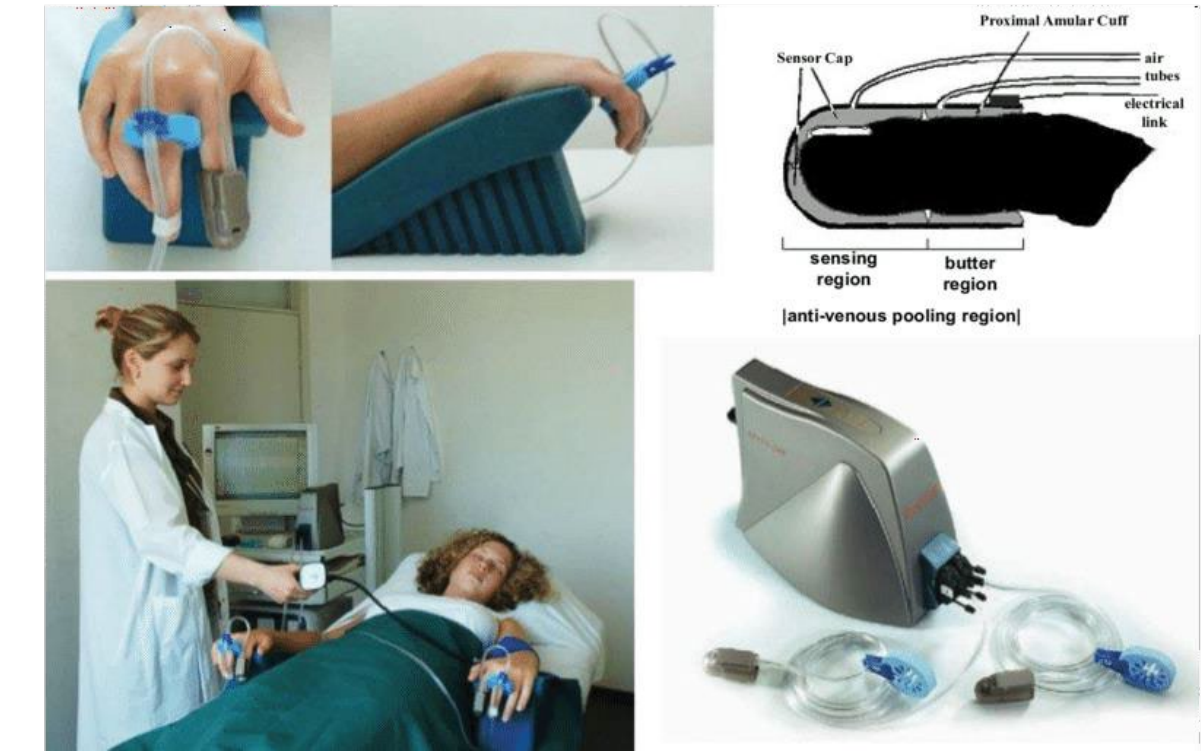


Figure 1. The Endo-PAT device.

16.2 Protocol

16.2.1 Subject Preparation

1. Prior to the study, ensure the patient has fasted for at least 4 hours, and has refrained for at least 8 hours from caffeine, tobacco, vitamins or medications that might affect vascular tone. The patient may wish to use the restroom prior to the study.
2. The Endo-PAT study will be conducted in a quiet, dimly lit, temperature-controlled exam room to reduce fluctuations in vascular tone.
3. Cell phones or paging devices should be silenced, and restrictive clothing that could interfere with blood flow to the arms should be removed. The patient should also remove watches, rings, or other jewelry on the hands or fingers.
4. Inspect the patient's fingers for any deformities or injuries that could affect the study. Do not place the probes on a finger that is cut or injured. Fingernails should not extend more than 5mm or 1/5 of an inch beyond the tip of the finger tissue. Trim or file fingernails if necessary to avoid damaging the internal membranes of the PAT probes and displacing the finger from the sensing region of the probe.

5. The index finger is recommended for the study; however, if this finger is unsuitable, a different digit (except the thumb) may be used, as long as the same finger is used on both hands.
6. The patient should be supine and comfortable for 15 minutes so as to attain a cardiovascular steady-state. Place the two arm supporters along each of the patient's sides.
7. Measure the blood pressure using the control arm (the arm that is not occluded during the Endo-PAT study.
8. Place a blood pressure cuff on the arm to be occluded during the Endo-PAT study. Apply the cuff snugly, but without excess pressure. Do not inflate the cuff at this time

16.2.2 Prepare the Endo-PAT System for Study

1. Launch the Endo-PAT 2000 software and click the "Patient Information" icon on the tool bar to create a new patient file.
2. Complete the Patient Information dialog box, including patient ID, name (optional), age, gender, height, weight, systolic and diastolic blood pressures. Optional fields allow for free text comments. Select your name from the pre-defined list in the Patographer name field. If your name has not been inserted add it the list and select.
3. Select two new PAT probes and connect to the pneumo-electrical tubing. To connect the probes, insert the connector tab into the probe slit and gently press the connector onto the probe until it clicks into place.
4. Place the connected probes into the sockets of the arm-supports and press the "Deflate" button on the top of the Endo-PAT 2000 device.

16.2.3 Conduct of the Endo-PAT Study

1. Place the patient’s index fingers completely into the probes, confirm with the patient that he or she can feel the very end of the probes, and press the "Inflate" button on the top of the Endo-PAT 2000 device.
2. Place a foam anchor ring at the base of the adjacent middle finger. Ensure that the foam ring and the PAT sensor do not touch. Otherwise the ring may mechanically interfere with the sensor.
3. Create an approximately 7-10cm loop with the pneumo-electrical tubing. The loop should extend from the PAT sensor and return to the foam ring on the adjacent finger while the rest of the tubing that connects to the EndoPAT device is pointing out tubing to the tip of the finger.
4. Position the patient's arms so the forearms are supported on the arm supports and the fingers dangle freely off the edge of the support. Make sure the probes are not in contact with any object, including the arm support, foam ring, tubing, the mattress or another finger.

5. Ask the patient to refrain from moving the fingers, as this will create mechanical artifacts. It is important for the patient to be relaxed throughout the study. Explain to the patient that during the test you will inflate the arm cuff, and during that time they may feel some discomfort, numbness, or tingling.

6. Click the "Standby" icon on the Endo-PAT's computer interface. Adjust the time base to 1 minute and adjust the signal gain on the screen to maximize signal clarity. Inspect the tracings of the PAT signals from the two probes to confirm that they are free of artifactual signals. If artifactual signals are present, veriify that the probes are not touching anything and that the patient is not moving the fingers.

7. To begin the study, click the "Go" icon on the computer interface. Start the stopwatch, by clicking the "Start/Stop Timer" icon. This will initiate a five minute count down for the baseline recording period. After five minutes, stop the stopwatch by clicking the "Start/Stop Timer" icon.

8. Tell the patient that you are going to inflate the cuff for the occlusion phase and that he or she should stay relaxed and not move the fingers.

9. Rapidly inflate the blood pressure cuff to a supra-systolic pressure of 60mmHg above the patient's systolic pressure or 200mmHg, whichever is higher and start the stopwatch again. Complete cessation of blood flow to the hand is verified by the absence of a PAT signal from the occluded arm. To confirm occlusion increase the gain on the screen of the channel of the occluded side to 20,000 while keeping the gain of the contra-lateral side constant. Decrease the time base of both channels to 30 seconds. Verify that you do not observe any signals at a periodicity that matches the signal from the control arm as this indicates an incomplete occlusion. If this is the case then further inflate the cuff until no signals are seen. The cuff may be inflated to a maximum of 300mmHg.

10. This will initiate a five minute count down for the arterial occlusion recording period. Toward the end of the occlusion period tell the patient you are going to release the cuff and that they should continue to refrain from moving their fingers. After exactly five minutes, deflate the cuff abruptly as quickly as possible and stop the stopwatch by clicking the "Start/Stop Timer" icon.

11. Click the "Start/Stop Timer" icon again to initiate a five-minute post occlusion recording period. Stop the timer after five minutes and click the "Test Stop" icon to complete the study. The probes will automatically deflate.

12. Remove the probes, tape, and foam rings from the patient's fingers and disconnect the PAT probes from the pneumo-electrical tubing. Discard the used probes.

16.2.4 Review and Analysis

1. Load the study file to the screen using the load icon. To run the automatic analysis, click the "magician stick" icon. The occlusion period will be highlighted in blue and the test result will be displayed, including the Reactive Hyperemia Index (RHI) and Heart Rate (HR), in the right-hand column of the screen.

2. To review additional data, including study parameters, calculated variables, patient information, and measures of signal quality, click the "Open Results of Last Calculation" icon. This will open a spread sheet with study parameters and results for all analyses performed to date, with the last line in the table containing data from the most recent analysis.

16.3 Representative Results

A representative Endo-PAT screen of a study performed on an individual with normal endothelial vasodilator function is shown in Figure 2 and a representative screen performed on an individual with endothelial vasodilator dysfunction is shown in Figure 3.

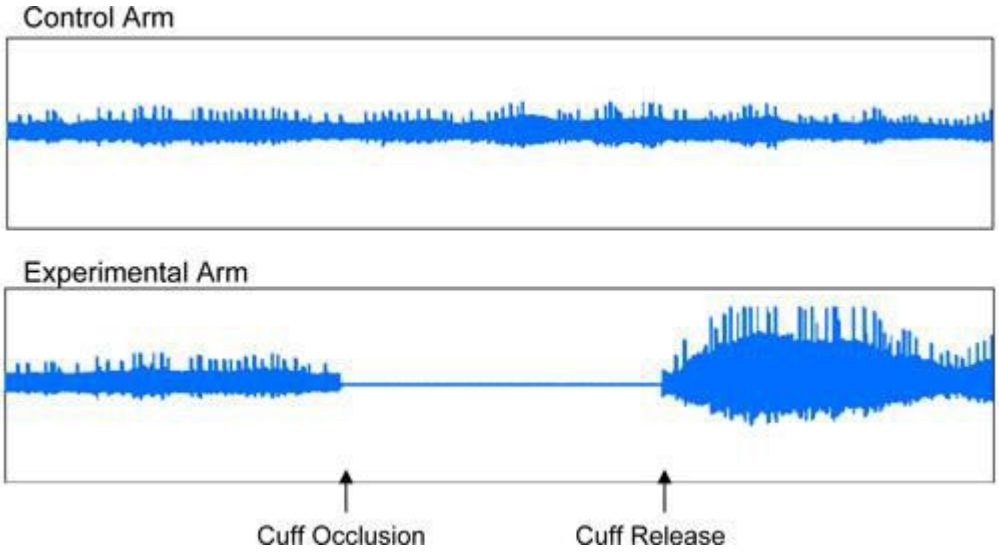


Figure 2: Normal Endothelial Vasodilator Function. Representative recording of an individual with normal endothelial vasodilator function, characterized by an increase in the signal amplitude after cuff release relative to baseline.

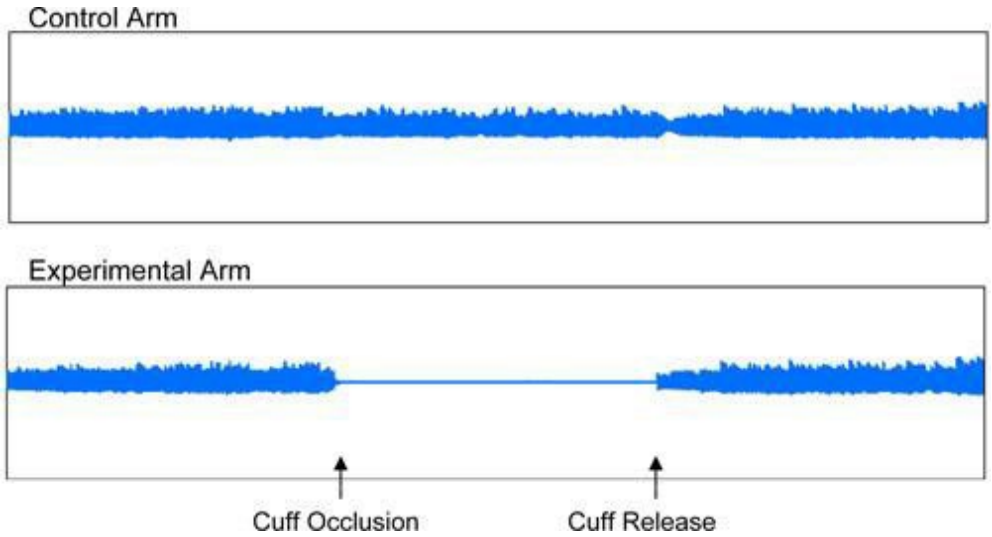


Figure 3: Abnormal Endothelial Vasodilator Function. Representative recording of an individual with abnormal endothelial vasodilator function, characterized by lack of increase in the signal amplitude after cuff release relative to baseline.