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(54) Title: PLATELET FUNCTION ANALYSIS FOR ADVERSE CARDIOVASCULAR EVENTS

(57) Abstract: The present invention relates to methods, compositions, assays and kits for identifying individuals with elevated risk of experiencing an adverse cardiovascular event after coronary or peripheral arterial bypass graft surgery. In particular, the invention relates to a method for identifying an individual at elevated risk for vein graft occlusion after coronary artery or peripheral bypass graft surgery comprising conducting a platelet function analysis.



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PLATELET FUNCTION ANALYSIS FOR ADVERSE CARDIOVASCULAR EVENTS

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Background

Coronary artery bypass (CABG) surgery is frequently used to treat patients with coronary artery disease who fail medical therapy or who are not candidates for percutaneous revascularization. Autologous vein grafts (VGs) are the most widely used conduits for CABG surgery, with nearly 1 million implanted annually in the US. Compared to internal mammary artery grafts, VG used for CABG surgery suffer significantly higher failure rates which limit their clinical efficacy.

Based on historic data, 8-12% of VG used for CABG surgery occlude within the first post-operative month (2). One and 10-year occlusion rates are 20-30% and 50-60% respectively, whereas the 10-year occlusion rate of internal mammary grafts is <10% (3,4). The disparity in patency has profound clinical implications. Patients who suffer VG occlusion within 2 years of CABG have a significantly worse long-term clinical outcome than patients with patent VG (5). Furthermore, patients who receive internal mammary grafts survive 10 to 30% longer and suffer significantly less angina than patients who receive only VG (6,7).

The major cause of early VG occlusion (*i.e.*, within the first 12 months of surgery) is thrombosis, whereas the predominant cause of VG occlusion beyond the first post-operative year is accelerated atherosclerosis (8,9). Early VG thrombosis is a multifactorial process involving both local and systemic factors. Recognized anatomic risk factors for early vein graft occlusion include small target vessel size and non-left anterior descending (LAD) coronary artery target vessel location (10,11). Systemic risk factors for early VG thrombosis are poorly defined. Though it is recognized that CABG surgery patients appear to be hypercoagulable in the early post-operative period, its relationship to VG thrombosis has not been firmly established (12-15). It has been well established that post-operative treatment with aspirin reduces both the rate of early VG thrombosis and improves survival after CABG surgery (16,17). It is for this reason that

the current ACC/AHA guidelines recommend administering aspirin within 24 hours of CABG surgery and continuing it for at least 1 year (18).

Aspirin is the most widely prescribed agent for established cardiovascular disease and has been shown to reduce recurrent cardio-vascular events by 34% and all-cause mortality by 18% (19). Some patients, however, may not derive full benefit from aspirin's antiplatelet effects, a concept referred to as aspirin "non-responsiveness", "insensitivity" or "resistance".

There is currently no accepted method to accurately identify patients at high risk for early vein graft failure and adverse clinical outcomes after CABG surgery even with aspirin therapy. There is a need to identify these patients so as to target them for alternative or intensive therapy to prevent VG.

The present invention provides assays and methods for identifying subjects at elevated risk for early vein graft failure and adverse outcome after CABG surgery utilizing shear-dependent platelet hyper-reactivity, aspirin-insensitive thromboxane generation, or both as independent and synergistic risk factors. These same methods can also be applied to a much broader population of patients with established or suspected for cardiovascular disease for identifying those at elevated or high risk for future adverse events.

Brief Description of the Drawings

Figure 1. Pathways of thromboxane production and the antiplatelet effect of aspirin. Dotted arrows denote "bypass" of aspirin's inhibition of COX-1. Adapted from Hankey and Eikelboom (1).

Figure 2. Multidetector CT angiographic reconstruction demonstrating the appearance of a patent saphenous VG (SVG) to the left anterior descending diagonal coronary artery (SVG-LADD), a patent left internal mammary graft to the left anterior descending coronary artery (LIMA-LAD) and an occluded SVG to the right coronary artery (SVG-RCA). The 3-dimensional whole-heart reconstruction of the whole heart is shown at left and the 2-dimensional reconstruction of the SVG-LADD is shown at right.

Figure 3. Incidence of aspirin resistance in patients on aspirin monotherapy 3 days after CABG and after 6 months by assays with defined thresholds for aspirin responsiveness. (*P =0.03, # P <0.001.)

Figure 4. Frequency of VG occlusion in 229 patients on aspirin monotherapy stratified both by quartile of PFA-100 CADP CT (A) and quartile of UTXB₂ (B). (P <0.005 by ANOVA for all groups.)

Figure 5. Frequency of VG occlusion in patients on aspirin monotherapy stratified by quartile of PFA-100 CADP CT and by upper quartile of UTXB₂.

Figure 6. Correlation between 11-dehydro-TXB₂ levels measured using the Esoterix and Corgenix assays.

Figure 7. Association between VG occlusion and tertiles of UTXB₂ levels determined by the Esoterix (A) and Corgenix (B) assays in the same urine samples.

Figure 8. (8A). CTs for CEPI and CADP agonist cartridges stratified by citrate concentration. Median values with interquartile ranges (boxes), 5% and 95% confidence intervals (bars) and individual outliers are shown. (8B) Percent of patients with low CTs by CEPI, CADP and both agonist cartridges stratified by citrate concentration.

Description

Described herein are methods and assays for identifying a subject at elevated risk for an adverse cardiovascular event.

The individual may be a human or non-human mammal. As used herein, the terms "subject", "patient", and "individual" are used interchangeably.

In a particular aspect, the invention relates to a method for identifying a subject at elevated risk for an adverse cardiovascular event comprising: carrying out one or more assays. In one embodiment, the assay comprises conducting a platelet function analysis on a sample of blood from the subject to determine the degree of platelet reactivity wherein if the degree of platelet reactivity is elevated, the patient is at risk of a cardiovascular event. In yet another embodiment, the assay comprises measuring thromboxane generation in the subject on aspirin, wherein if there is persistent thromboxane generation despite aspirin therapy, the patient is at risk of cardiovascular event. In yet another embodiment, the assay comprises the combination of both conducting a platelet function analysis on a sample of blood from the subject to determine the degree of platelet reactivity along with measuring thromboxane generation in the subject on aspirin, wherein if the degree of platelet reactivity is elevated and/or there is persistent thromboxane generation despite aspirin therapy, the patient is at risk of cardiovascular event.

By "elevated", "increased" or "high" risk is meant a measurable increase in risk, especially a statistically significant increase in risk, over a control group, e.g. a group of patients having an "average" risk of a particular event, or an elevated risk over the entire population of patients within a particular treatment group.

5 In yet another aspect, the method comprises conducting a platelet function analysis on a sample of blood from a patient to determine the degree of platelet reactivity, e.g., shear-dependent platelet reactivity, and measuring thromboxane, e.g., thromboxane A₂, generation in a patient on aspirin, wherein if the degree of platelet reactivity, e.g., shear-dependent platelet reactivity, is elevated and/or there is persistent thromboxane generation A₂ despite aspirin
10 therapy, then the patient is at risk of cardiovascular event.

A cardiovascular event may include an adverse event or condition related to a cardiovascular disorder or disease, coronary artery disease, cardiac surgery, peripheral bypass graft surgery, coronary artery bypass (CABG) surgery, or an adverse clinical outcome after CABG surgery, failure after CABG surgery, internal mammary artery graft failure, vein graft
15 failure, autologous vein grafts, vein graft occlusion, or vein graft occlusive due to thrombosis, or accelerated atherosclerosis.

The term "cardiovascular disease" refers to a class of diseases that involve the heart and/or blood vessels (arteries and veins), i.e., any disease that affects the cardiovascular system.

In a particular embodiment, the adverse event may be related to early failure of arterial
20 grafts related to thrombosis. In another embodiment, the cardiovascular event may be a cardiovascular disease, cardiovascular death, myocardial infarction, need for coronary revascularization, stroke, graft occlusion or failure, heart failure or pathologic thrombotic/thromboembolic event. In yet another aspect, the cardiovascular event is an adverse condition related to coronary artery bypass (CABG) surgery, i.e., vein graft occlusion.

25 In another embodiment of the invention, the subject may be at risk of an adverse cardiovascular event after coronary artery or peripheral bypass graft surgery. In yet another aspect, the subject is at risk of early vein graft occlusion after coronary artery or peripheral bypass graft surgery.

The method of invention, e.g., the assay, test or procedure, may be carried out or
30 conducted pre- (before or prior to) or post- (after) surgery, e.g., coronary artery or peripheral bypass graft surgery. The assay may be conducted, minutes, hours, days, weeks or months

before the surgery, e.g., one day prior to surgery or two, three, four, five or six days, one week, two weeks, three weeks, four weeks, or a month prior to surgery.

The assay may be conducted after surgery, e.g., days, weeks, months or years. For example, the method may be conducted at four, five or six days, or one, two, three or four weeks, or one, two, three, four, five, six, seven, eight, nine, ten, eleven or twelve month(s) after post coronary artery bypass surgery, or at least monthly or yearly post coronary artery bypass surgery. The assay may be done as part of a routinely or regularly schedule medical check up.

As used herein the blood sample from the patient may be whole blood or platelet-rich plasma.

An assay as used herein refers to a procedure for testing and/or measuring the activity of a substance, compound, drug, cell, enzyme protein or other biological component or biochemical in an organism or organic sample.

Aspirin non-responsiveness, insensitivity or resistance as used herein can be defined both clinically and biochemically. Clinically, 8-18% of patients on aspirin suffer a recurrent cardiovascular event within two years and therefore may be considered to be resistant (20). The main biologic effect of aspirin is to irreversibly inhibit platelet cyclooxygenase-1 (COX-1), a critical enzyme in the thromboxane A₂ (TXA₂) synthesis pathway (Figure 1). Platelets synthesize and release TXA₂ in response to a number of stimuli (i.e., collagen, ADP, and thrombin) where it acts to amplify platelet aggregation and cause vasoconstriction. Aspirin resistance can therefore be defined biochemically as the failure to inhibit platelet TXA₂ production (1).

Potential causes of aspirin non-responsiveness, insensitivity or resistance included: 1) Patient non-compliance, inadequate dosing, or interference by non-steroidal anti-inflammatory drugs may result in incomplete COX-1 inhibition (21). 2) Because the half-life of aspirin in the blood is only ~20 minutes, patients with rapid platelet turnover may have incomplete platelet inhibition with once-a-day dosing. 3) Genetic polymorphisms in the COX-1 enzyme or other downstream pathway proteins could reduce sensitivity to aspirin. 4) Non-TXA₂ pathways may predominate in mediating platelet activation. Although the TXA₂ pathway is the sole mediator of arachidonic acid-induced platelet activation, it is but one of the mediators of activation in response to weak agonists such as ADP and epinephrine and contributes very little to activation in response to strong agonists such as thrombin and collagen. Depending on the stimulus, inhibition of the TXA₂ pathway may have only a limited effect on preventing platelet activation.

5) TXA₂ can be generated by non COX-1-dependent pathways. For example, immature platelets and megakaryocytes contain small amounts of the COX-2 enzyme that is ineffectively inhibited by aspirin. TXA₂ synthesis can also occur in circulating mononuclear cells and endothelial cells via COX-2 induction by inflammatory mediators. These cells can either generate TXA₂ directly or transfer its precursors, PGG₂ and PGH₂, to platelets which can complete the pathway below the level of the COX-1 block (Figure 1) (22).

While a number of laboratory assays have been used to identify aspirin non-responsiveness, insensitivity or resistance, there is currently no consensus on a "gold standard." Some assays are accepted as indicative of non-responsiveness, insensitivity or resistance to aspirin.

Platelet reactivity or function can be determined in a variety of manners, e.g. shear-dependent platelet reactivity, platelet aggregation, whole blood platelet aggregation, optical density platelet aggregation, flow method (VASP) and other methods. For example, see United States Published Patent Application No. 20080009024, published January 10, 2008 (Christie) and Methods for Evaluation of Platelet Function. Transfusion and Apheresis Science, Lindahl, T. Ramston, S.; September 2009, which are both hereby incorporated by reference in their entirety.

The role of platelets in mammalian physiology is primary role is in promoting hemostasis. When exposed to a damaged blood vessel, platelets will adhere to an exposed sub-endothelial matrix. Following the initial adhesion, various factors released at the site of injury such as thrombin, ADP and collagen activate the platelets. Once platelets are activated, a conformational change occurs in the platelet glycoprotein GPIIb/IIIa receptor allowing it to bind fibrinogen and/or von Willebrand factor. It is this binding of the multivalent fibrinogen and/or von Willebrand factor molecules by GPIIb/IIIa receptors on adjacent platelets that results in the recruitment of additional platelets to the site of injury and their aggregation to form a hemostatic plug or thrombus.

As used herein, the terms "activity" or "reactivity" may be used interchangeably to relate to a change in platelet function and activation in relation to an established cut-off-value. These terms may be used in conjunction with platelet function analysis. For example, platelet hyperactivity or hyper-reactivity in the context of the present disclosure can mean that the time to platelet plug formation using the PFA-100 (CADP cartridge), as described below, in a given patient's platelet-containing samples is below an established cut-off value.

In a particular embodiment, the platelet-containing sample is evaluated for platelet hyperactivity. The platelet-containing sample is any sample from an individual that contains platelets that can be evaluated for hyperactivity. In many instances the sample is whole blood or platelet rich plasma. The particular form of the platelet-containing sample may vary depending on the nature of the method for determining platelet hyperactivity.

Platelet hyperactivity in the context of the present disclosure can mean that the time to platelet plug formation in a given patient's platelet-containing samples is below an established cut-off value.

The extent that the platelets exhibit hyper-reactivity is related to the risk of a patient experiencing an adverse cardiovascular event. Generally, platelet hyper-reactivity, having a platelet plug formation time of less than a certain point, e.g., 74 seconds. This means that the patient has an elevated or increased risk of experiencing a future cardiovascular event four to seven times more likely than if the platelet plug formation time is greater than or equal to 74 seconds.

In some embodiments platelet hyper-reactivity is evaluated using a high shear force test method or high shear platelet function test. In some embodiments of such a method, a platelet-containing sample from a patient is drawn under vacuum through a capillary and platelets are activated by means of shear force.

As a result of being subjected to a high shear force in the capillary, the platelets become activated. Activation of the platelets can mean that a conformational change occurs in the platelet glycoprotein GPIIb/IIIa receptor allowing it to bind fibrinogen and/or von Willebrand factor. It is this binding of the multivalent fibrinogen and/or von Willebrand factor molecules by GPIIb/IIIa receptors on adjacent platelets that results in the recruitment of additional platelets to a site of injury and their aggregation to form a hemostatic plug or thrombus.

In some embodiments a membrane comprises a platelet aggregation stimulator, which is an agent that promotes aggregation of the platelets. Platelets are known to aggregate under a variety of conditions and in the presence of a number of different reagents. Platelet aggregation is a term used to describe the binding of platelets to one another. The phenomenon can be induced by adding aggregation-inducing agents to platelet-rich plasma (PRP) or to whole blood. Platelet aggregation in vitro depends upon the ability of platelets to bind fibrinogen and/or von Willebrand factor to their surfaces after activation by an aggregation-inducing agent such as ADP or collagen.

The amount of the platelet aggregation stimulator on the membrane is that which is effective or sufficient to promote aggregation of the platelets in the sample under the conditions of the present methods. The amount employed depends on the nature of the platelet aggregation stimulator, the nature of the sample and the like. The amount of platelet aggregation stimulator is about 1 µg to about 100 µg, about 5 µg to about 80 µg, about 10 µg to about 70 µg, about 20 µg to about 60 µg, or about 30 µg to about 50 µg, and the like. In one embodiment the membrane comprises 50 µg of adenosine-5'-diphosphate and about 2 µg of collagen. By "diffusively bound" is meant that the platelet aggregation stimulator is bound to the membrane so that it will be removed from the membrane during the present method, usually, upon contact with, or exposure to, a sample being tested. The platelet aggregation stimulator may be incorporated in the membrane as a result of an ability to penetrate the membrane and, in some embodiments saturate the membrane, thereby forming a film on the membrane as well as being incorporated therein.

In some embodiments the methods disclosed herein may be carried out using apparatus and reagents disclosed in U.S. Patent No. 5854076, the relevant disclosure and figures thereof being incorporated herein by reference.

In a particular aspect, the assay comprises conducting a platelet function analysis on a sample of blood from the subject to determine the degree of shear-dependent platelet reactivity wherein if the degree of shear-dependent platelet reactivity is elevated, the patient is at risk of a cardiovascular event. "Shear-dependent" platelet reactivity as used here in refers to a determination of platelet function under flow conditions or shear forces.

Platelet aggregometry: Platelet aggregation is traditionally measured in either platelet-rich plasma or whole blood by changes in light transmission or electrical impedance in response to agonists such as arachidonic acid, ADP, epinephrine or collagen. Because platelet aggregation in response to arachidonic acid is solely dependent on COX-1 activity, this is the most sensitive indicator of an aspirin effect. In a study of 1282 patients, low-dose aspirin caused complete suppression of arachidonic acid-induced platelet aggregation in over 95% of patients. (23). When aspirin was added directly to these same platelet samples to compensate for possible non-compliance or under-dosing, 99.5% of patients exhibited complete suppression, suggesting that aspirin can effectively block COX-1 activity in the vast majority of individuals. As other agonist (e.g. ADP, epinephrine, thrombin and collagen) stimulate platelet aggregation predominantly through non-TXA₂ pathways, they are less useful in identifying aspirin's failure to specifically

inhibit COX-1 but may be indicative of the relative important of the TXA₂ pathway in mediating platelet aggregation. Depending on the agonist and thresholds, aspirin resistance assessed by aggregometry is reported to occur in 5-35% of individuals. (20).

Rapid Platelet Function Analyzer-ASA (RPFA-ASA): The Ultegra RPFA-ASA (VerifyNow Aspirin® Accumetrics, San Diego, CA) is a point-of-care device that turbidimetrically measures platelet-induced aggregation of fibrinogen-coated beads incubated with whole blood in response to stimulation with arachidonic acid or propyl-galate. The results are expressed in aspirin resistance units (ARU), with values ≥ 550 being indicative of non-responsiveness to aspirin therapy. By this assay, the incidence of aspirin resistance is approximately 20%, with those resistant patients being more likely to have coronary artery disease and suffer a higher incidence of myonecrosis after percutaneous coronary intervention than aspirin sensitive patients (29, 30).

In a particular embodiment of the invention, the shear-dependent platelet reactivity can be measured using a variety of techniques, for example one using collagen and ADP. The Platelet Function Analyzer-100® (PFA-100) (Siemens Healthcare Diagnostics Inc., Deerfield, IL) is a point-of-care device that simulates in vivo shear-dependent platelet activation by propelling blood through a small orifice coated with a combination of collagen and either ADP or epinephrine. As platelet activation and aggregation occurs around the orifice, this device assesses agonist-induced platelet aggregation under high shear by measuring the closure time (CT) of a membrane aperture by the formation of a platelet plug. The degree of platelet reactivity is inversely proportional to CT. The two agonist cartridges currently available are the collagen/epinephrine (CEPI) agonist cartridge, which detects the antiplatelet effects of aspirin, and collagen/ADP (CADP) agonist cartridge, which assesses global platelet reactivity that is not affected by aspirin administration. In a particular embodiment, the collagen-ADP assay can be used to assess risk in someone not on aspirin.

In one particular embodiment, the samples from blood collected are in 3.8% or 3.2% citrate. In a preferred embodiment, the collected blood sample is in 3.8% citrate.

With either agonist cartridge, prolongation of the closure time beyond the normal range indicates inhibited or impaired platelet function. The normal closure time range for healthy individuals is approximately 71-118 seconds using collagen/ADP (CADP) agonist cartridge. For the collagen/epinephrine (CEPI) agonist cartridge normal closure time is 74-193 seconds,

whereas in individuals taking aspirin the closure time with this cartridge is typically prolonged >300 seconds.

In one embodiment of the invention, the elevated degree of platelet reactivity or hyperactivity with either agonist cartridge is indicated by a lower closure time.

5 By "elevated" degree of platelet reactivity, e.g., shear-dependent platelet hyper-reactivity, is meant a measurable increase in the degree of platelet reactivity, e.g., shear-dependent platelet hyper-reactivity, especially a statistically significant increase, over a control group, e.g. a group of patients having an "average" or "mean" change of a particular event, or an elevated degree over the entire population of patients within a particular treatment group. For
10 example an "elevated" degree may be one that is below an established normal range for given patient population, e.g., below an established cut-off value. For example, an established cut-off value may be below 71 seconds for the CADP cartridge.

The risk of vein graft occlusion proportionally increases as closure time decreases in linear fashion. The closure time can be calculated, calibrated or set in a variety of ways. Risk
15 can be assessed using a median value to calculate risk using binary variables or incrementally using population quartiles. For example, a low closure time may be below 150, 125, 100, 90, 88, 74, 73, 72 or 71 seconds. High closure time can be indicative of a reduced degree of shear-dependent platelet reactivity and a reduced risk of adverse conditions. High closure time may include, e.g. above 168, 170, 180, 190, 200, 250, 300 seconds.

20 In yet another embodiment, the closure time rate can be proportional to the risk rate, with progressively reduced closure times associated with progressively increasing risk of cardiovascular disease or vein graft occlusion. For example, a subject patient having a lower closure time rate of equal to or less than, e.g., 88 seconds (as would be calculated based on the median in a patient population) has a more than 2.5-fold higher frequency of having
25 cardiovascular disease or vein graft occlusion than a patient with closure time rate of greater than, e.g., 88 seconds. Closure times may be calculated and set to be selected from a specific number from the range of 46 to 75 seconds, 78 to 88 seconds, 89 to 105 seconds, or 106 to 300 seconds, and could be used to predict the likelihood, frequency or amount of vein graft occlusion, as more than 25%, 20%, 15% or 10%, respectively. In another embodiment, one can
30 extrapolate from multivariate analysis, for every 20-second decrease in the CADP of closure time, the risk of vein graft occlusion increases 50%.

As used herein, one condition associated with risk is aspirin-insensitive thromboxane generation, i.e. persistent thromboxane generation despite aspirin therapy (which inhibits COX-1 mediated platelet thromboxane generation). Thromboxane, e.g., thromboxane A₂, is a short-lived substance that is quickly degraded to more stable metabolites, for example thromboxane B₂. The thromboxane B₂ metabolite is further metabolized into a number of other metabolites. Thromboxane B₂ (TXB₂) metabolites include, e.g., 11-dehydro-thromboxane B₂, 2,3-dinor-thromboxane B₂ and 11-dehydro-2,3-dinor- thromboxane B₂. Other examples are provided in Roberts, et al, "Metabolism of thromboxane B₂ in man. Identification of twenty urinary metabolites", J Biol Chem. 1981 Aug 25;256(16):8384-93, hereby incorporated by reference in its entirety. Aspirin-insensitive thromboxane, e.g., thromboxane A₂ (TXA₂), generation can be measured in a number of ways. One method is to measure the urinary concentration of its stable metabolite, e.g., 11-dehydro-thromboxane B₂ (11-dehydro-TXB₂). The metabolite, e.g., 11-dehydro-TXB₂, can also be measured in the serum or plasma. The metabolite, e.g., 11-dehydro-TXB₂, accumulates in the urine and when normalized to urine creatinine provides a measurement of total body TXA₂ generation. Because 95% of normal individuals on chronic aspirin therapy have a urine 11-dehydro-TXB₂/creatinine ratio (UTXB₂) <400 pg/creatinine as measured by the ASPIRINCheck® assay, this value has been used as the threshold for defining aspirin insensitivity or resistant (31).

In one embodiment, the assay comprises measuring thromboxane A₂ generation in a patient on aspirin, and if there is persistent thromboxane generation despite aspirin therapy, then the patient is at risk of cardiovascular event.

By "persistent" thromboxane generation is meant as a measurable increase in thromboxane generation despite aspirin therapy, especially a statistically significant increase, over a control group, e.g. a group of patients having an "average" or "mean" change of a particular event, or an elevated degree over the entire population of patients within a particular treatment group. For example, a persistent amount may be an increase of thromboxane generation above 400 pg/mg creatinine as measured using the ASPIRINCheck® assay or more than 1500 pg/mg creatinine as measured using the Corgenix version of the assay.

In a particular aspect, the metabolite is 11-dehydrothromboxane B₂.

There are a variety of ways to determine thromboxane generation, e.g., thromboxane A₂, generation e.g., by measuring the metabolite using Enzyme-linked immunosorbent assay (ELISA) or ELISA-based assays or using gas-liquid chromatography, mass spectroscopy or

other methods. For example, see United States Published Patent Application Nos. 2004/0126866 (Yusuf et al.) and 2007/0202556 (Geske et al.), and United States Patent Nos. 504735 (Foegh et al.), 6967083 (Ens) and 7081347 (Yusuf et al.), which are all hereby incorporated by reference in their entirety.

5 An ELISA-based assay may include e.g. one previously offered through Esoterix, Austin, TX (ASPIRIN*Check*®) or another offered through Corgenix Medical Corporation, Broomfield, CO. (AspirinWorks®).

10 In a particular embodiment, the patient may be on aspirin therapy, e.g., wherein the sample of urine is collected after the administration of aspirin. Administration of aspirin may be on a daily or a regular basis, e.g., within two to three days. Administration of a dose of aspirin may be in an amount of equal or greater than 50 mg. In another embodiment, the patient is provided a single or multiple dose(s) of aspirin prior to measuring the thromboxane.

 In another embodiment, an "increased", "higher" or "elevated" level of thromboxane metabolite, e.g., TXB₂, in the urine is indicative of an elevated risk of adverse conditions.

15 In yet another embodiment, the elevated level or concentration of thromboxane metabolite can be proportional to the risk rate, with progressively increase in levels associated with progressively increasing risk of cardiovascular disease or vein graft occlusion. Elevated levels may be calculated, calibrated or set in a variety of ways. The set level may also depend on the type of assay used. For example, an elevated or increased level can be an 11-dehydro-thromboxane B₂/creatinine ratio of more than 449 pg/mg creatinine as measured by an Esoterix
20 ELISA assay or of more than 681 pg/mg creatinine as measured by a Corgenix ELISA assay. Set levels may include, e.g., 11-dehydro-thromboxane B₂/creatinine ratio of more than 400, 425, 449 450, 500, 550, 600, 650, 681, 700 pg/mg. The level may be calculated and set to be selected from a specific number from the range of 104 to 233 pg/mg, 234 to 330 pg/mg, 331 to 448
25 pg/mg, or 449 to 2500 pg/mg, and could be used to predict the likelihood, frequency or amount of vein graft occlusion, as more than 10%, 15%, 15% or 25%, respectively.

 In another embodiment, the level may be calculated and set to be selected from a specific number from less than 271 pg/mg as 19-20 % occlusion, from the range of 271 to 439 pg/mg as 20-21% occlusion, or more than 439 to about 38% occlusion (using Esoterix) or more than 571
30 pg/mg as about 22% occlusion, from the range of 576-940 pg/mg as about 23% occlusion, or more than 940 to more than 30% occlusion (using Corgenix).

The level of thromboxane metabolite can be proportional to the risk level, wherein a higher level is associated with a progressive increase in the frequency of risk of cardiovascular disease or vein graft occlusion.

5 The thromboxane metabolite may be proportional to the risk level, wherein a higher level is associated with a progressive increase in the frequency of risk of cardiovascular disease or VG occlusion. For example, a patient having a level of urinary 11-dehydro-thromboxane B₂ of more than 449 pg/mg creatinine as measured by an Esoterix ELISA assay has a more than 2.5-fold higher increase of vein graft occlusion than a patient with a lower amount. In another embodiment, one can extrapolate from multivariate analysis, for an increase in urine 11-dehydro-TXB₂ by the Esoterix assay from 300 to 812 pg/mg creatinine, the risk of VG occlusion increases by 73%.

10 In another embodiment, an assay for conducting a platelet function analysis to determine the degree of shear-dependent platelet reactivity and an assay for measuring thromboxane metabolite levels from a urine sample, are used together to provide a synergistic effect for identifying the risk of an adverse cardiovascular event. For example, on average, a patient having a level of urinary 11-dehydro-thromboxane B₂ of more than 449 pg/mg creatinine as measured by an Esoterix ELISA assay and a PFA-100 CADP CT of equal to or less than 88 seconds has nearly 7.0-fold higher risk of an adverse cardiovascular event than a patient with a level of 11-dehydro-thromboxane B₂ equal to or less than 449 and CADP CT more than 88 seconds.

20 In a particular embodiment, the invention relates to a method for identifying a patient or individual at elevated risk for an adverse cardiovascular event comprising: conducting a platelet function analysis on a sample of blood from a patient to determine the degree of platelet reactivity, and measuring thromboxane A₂ generation in a patient on aspirin, wherein if the degree of platelet reactivity is elevated and/or there is persistent thromboxane generation despite aspirin therapy, then the patient is at risk of cardiovascular event. The adverse cardiovascular event may be in patients with risk factors for cardiovascular disease, suspected cardiovascular disease or established cardiovascular disease. In patients undergoing coronary arterial or peripheral arterial bypass graft surgery the adverse cardiovascular event could occur either before or after surgery. In one aspect, the platelet reactivity is the shear-dependent platelet reactivity. In another aspect, the cardiovascular event is a cardiovascular disease, death, myocardial infarction, need for coronary revascularization, stroke, graft occlusion or failure,

pathologic thrombotic event, or heart failure. In yet another aspect, the cardiovascular event is an adverse condition, e.g., vein graft occlusion, related to coronary artery bypass (CABG) surgery or peripheral bypass surgery.

Kits with one or multiple components of the assay, are included in the present invention.

5 Such kits, in addition to the containers containing the multiple or unit doses of the assay, optionally include an informational package insert with instructions describing the use and attendant benefits of the assay components. In the kit the reagents can be provided in packaged combination in the same or separate containers, depending on the cross-reactivity and stability of the reagents, so that the ratio of reagents provides for substantial optimization of a signal from
10 the reporter molecule used in the detection system. The diagnostic kit can comprise in packaged combination one or more test cartridges comprising a capillary and membrane. The kit may also include other reagents as may be employed in the tests.

The invention also relates to a assay, kit or package for identifying a patient or individual at elevated risk for adverse a cardiovascular event, said kit containing: reagents for conducting a
15 platelet function analysis to determine the platelet reactivity, e.g., shear-dependent platelet reactivity, reagents for measuring thromboxane generation, e.g., metabolite levels from a urine sample, and optionally a script or instructions for determining whether the patient is at elevated risk for adverse cardiovascular events. The components of these kits can be as described above.

In another aspect, the invention relates to the use of an assay comprising platelet function
20 analysis to identify patients at elevated risk for adverse cardiovascular events prior to or after coronary artery bypass surgery. In yet another aspect, the invention relates to an assay comprising measuring the level of thromboxane generation, e.g., measuring a thromboxane metabolite in the urine, to identify patients at elevated risk for adverse cardiovascular events prior to or after coronary artery bypass surgery. In yet a further embodiment, the invention
25 relates to the use of the above assay to identify individuals at risk of adverse cardiovascular events to provide alternative or additional preventive therapy.

In another embodiment, the invention relates to a method of pre-operatively or post-operatively identifying a high-risk patient of adverse cardiovascular events and providing and modifying alternative therapy. In a particular embodiment, the alternative therapy comprising
30 using a pharmacological agent that would decrease shear-dependent platelet reactivity and/or aspirin insensitive thromboxane generation. Pharmacologic agents that could potentially be used to modify platelet reactivity or hyper-activity, e.g., shear-dependent platelet reactivity or

hyper-activity, and/or modify the risk associated with platelet reactivity or hyper-activity, e.g., shear-dependent platelet reactivity or hyper-activity, include for example; glycoprotein IIb/IIIa inhibitors, ADP-receptor antagonists, phosphodiesterase inhibitors, thrombin-receptor antagonist, inhibitors of vonWillebrand factor function, inhibitors of glycoprotein Ib-IX-V function, or modulators of ADAMTS13 activity.

Pharmacologic agents that could potentially be used to modify aspirin-insensitive thromboxane generation and/or modify the risk associated with aspirin-insensitive thromboxane generation, include for example; thromboxane synthase inhibitors, thromboxane receptor antagonists, antioxidants or omega-3 fatty acids.

As used herein, the singular forms "a", "an", and "the" include plural forms unless the context clearly dictates otherwise. Thus, for example, reference to "an assay" includes a plurality of such assay.

The invention is to be understood as not being limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

All publications mentioned herein, including patents, patent applications, and journal articles are incorporated herein by reference in their entireties including the references cited therein, which are also incorporated herein by reference.

The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

This application claims priority to U.S. provisional application nos. 61/101733, filed October 1, 2008 and 6/156237, filed February 27, 2009, which is hereby incorporated herein by reference.

EXAMPLES

Example 1

The Reductions in Graft Occlusion Rates Study

An observational study was conducted aimed at identifying novel risk factors for early
5 vein graft thrombosis after coronary artery bypass (CABG) surgery. As part of the study, over
3000 clinical and laboratory data elements were collected on each patient related to their
procedure and clinical course over the ensuing 5 years. Serum, plasma, DNA and urine samples
are collected preoperatively, 3 days, 6 weeks and 6 months postoperatively for selected assays
and the remainder stored for future analysis. Six months after CABG surgery, non-invasive
10 assessment was determined for vein graft patency using non-invasive multidetector computed
tomography coronary angiography (MDCTCA).

Figure 2 shows the results of the MDCTCA with a reconstruction demonstrating the
appearance of a patent SVG to the left anterior descending diagonal coronary artery (SVG-
LADD), a patent left internal mammary graft to the left anterior descending coronary artery
15 (LIMA-LAD) and an occluded SVG to the right coronary artery (SVG-RCA). The 3-
dimensional whole-heart reconstruction of the whole heart is shown at left and the 2-
dimensional reconstruction of the SVG-LADD is shown at right.

Example 2

The Reductions in Graft Occlusion Rates Study

A total of 368 patients were enrolled in the study. The study population is very
representative of patients undergoing isolated CABG surgery in the United States when
compared to data from the Society for Thoracic Surgeons National Database. Of the 368
enrolled patients with 642 SVGs, 6-month patency was determined in 297 (80.7%) patients and
25 6-month clinical endpoints were determined in 330 (89.7%) patients. Blood was collected in
3.8% sodium citrate.

One of the goals of the study was to determine if resistance to aspirin contributes to early
VG thrombosis and adverse clinical outcome after CABG surgery. To determine this, blood and
urine samples were collected 4 days and 6 months after surgery for characterization of platelet
30 function and assessment of aspirin response. Patients enrolled at other sites only underwent
assessment of platelet function at the 6 month visit. Platelet function was assessed by
aggregometry in response to stimulation with arachidonic acid (500 μ M), ADP (5-20 μ M),

epinephrine (50 :M), and collagen (1 :g/mL), with the PFA-100 (using both collagen/epinephrine [CEPI] and collagen/ADP [CADP] as agonist cartridges), by RPFA-ASA, and by measurement of UTXB₂. Patients received aspirin, 325 mg, beginning on post-operative day 1 and a 6-month supply of 325 mg enteric coated aspirin was supplied upon discharge.

5 Aspirin use was verified by review of the medical record and at 6 months by pill counts. Baseline pre-operative studies were not performed due to the variability of aspirin use prior to surgery and because of potential confounding effects on some assays of non-aspirin antiplatelet agents (e. g., IIb/IIIa inhibitors or ADP-receptor antagonists) given to patients with acute coronary syndromes. Six-month postoperative platelet studies were assumed to represent the
10 patient's baseline state.

Results

Incidence of Aspirin Resistance after CABG Surgery

The incidence of aspirin resistance was first determined both early after surgery (post-op
15 day 3-4) and after 6 months using assays with well-defined cut-off values for aspirin responsiveness. The percentage of patients with aspirin resistance varied greatly depending on the assay employed and with respect to the timing of the analysis. Figure 3 shows the incidence of aspirin resistance in patients on aspirin monotherapy 3 days after CABG and after 6 months by assays with defined thresholds for aspirin responsiveness (*P =0.03, # P <0.001).

20 By platelet aggregometry >95% of patients had complete suppression of aggregation to 500 :M arachidonic acid, indicating compliance with aspirin therapy and effective inhibition of platelet COX-1. In the early post-operative period, there was a very high degree of aspirin resistance as defined by PFA-100 CEPI (CT>193s) and by elevated UTXB₂ levels (≥ 400 pg/mg creatinine) which persisted in approximately 30% of patients 6 months after surgery. In contrast,
25 aspirin resistance as defined by RPFA-ASA (ARU ≥ 550) occurred in 10-12% of patients and did not change appreciably over time.

These results indicate that in the vast majority of patients, aspirin effectively inhibited platelet COX-1 activity, even early after CABG surgery. There were significant numbers of patients who continued to have substantial amounts of aspirin-insensitive thromboxane
30 generation.

Shear-dependent Platelet Hyper-reactivity and Aspirin-insensitive Thromboxane Generation Correlate with Early VG Occlusion after CABG Surgery

Assays of aspirin resistance and platelet reactivity were assessed both 3 days and 6 months after CABG surgery to demonstrate the correlation with VG outcome, assessed both on a per-patient and per-VG basis. When measured 3 days after CABG surgery no assay was found to correlate with SVG occlusion (data not shown). When measured 6 months after CABG surgery, however, occluded SVGs were associated with lower PFA-100 CADP CTs and higher levels of UTXB₂ compared to patent SVGs (Table 1).

Table 1. Relationship between platelet reactivity and ASA responsiveness, measured 6 months after CABG surgery, and 6-month SVG patency.

Assay	Per Patient				Per SVG			
	≥1 Occluded SVG	No Occluded SVG	N	P value	Occluded SVG	Patent SVG	N	P value
Platelet Aggregometry (Ω)								
Arachidonic Acid, 0.5 mM (median, IQR)	0 (0-0)	0 (0-0)	228	NS	0 (0-0)	0 (0-0)	495	NS
ADP, 5 μM (mean ± SD)	17.7 ± 5.5	17.8 ± 4.8	212	NS	17.5 ± 5.2	17.7 ± 4.7	463	NS
ADP, 10 μM (mean ± SD)	17.8 ± 4.8	17.7 ± 5.1	209	NS	17.5 ± 4.7	18.0 ± 4.9	457	NS
ADP, 20 μM (mean ± SD)	16.4 ± 5.3	17.1 ± 4.4	224	NS	16.1 ± 5.3	17.1 ± 4.7	483	NS
Epinephrine, 50 μM (mean ± SD)	5.20 ± 5.76	5.50 ± 6.45	227	NS	5.47 ± 5.59	5.23 ± 6.33	490	NS
Collagen, 1 μg/mL (mean ± SD)	15.1 ± 5.1	14.8 ± 5.2	228	NS	14.3 ± 5.6	14.9 ± 5.2	495	NS
PFA-100 CT (seconds)								
CEPI (median, IQR)	288 (245-300)	300 (257-300)	226	NS	288 (245-300)	295 (260-300)	491	NS
CADP (median, IQR)	80 (71-95)	92 (77-110)	228	0.001	80 (72-98)	88 (77-106)	492	0.0007
UTXB₂ (ln pg/mg creatinine; mean ± SD)	5.90 ± 0.62	5.77 ± 0.52	228	NS	5.98 ± 0.63	5.76 ± 0.55	495	0.0008
VerifyNow Aspirin (ARU; mean ± SD)	452 ± 55	460 ± 60	228	NS	450 ± 51	458 ± 60	496	NS

Because specific VG characteristics can variably influence graft patency within a given patient, we further explored whether PFA-100 C/ADP CT and urine 11-dehydro-TXB₂ were associated with the cumulative frequency of VG occlusion. Figure 4 shows frequency of VG occlusion in 229 patients on aspirin monotherapy stratified both by quartile of PFA-100 CADP CT (A) and quartile of UTXB₂ (B) (P < 0.005 by ANOVA for all groups).

Shear-dependent platelet hyper-reactivity, as measured by PFA-100 CADP CT, was significantly associated with a progressive increase in the frequency of VG occlusion: Patients in the lowest CT quartile (most reactive platelets) had >2.5-fold higher frequency of VG occlusion

than patients in the highest CT quartile (least reactive platelets). Likewise, the level of UTXB₂ was also significantly associated with the frequency of VG occlusion (Figure 4B): Patients in the highest quartile had a 2.5-fold increase in VG occlusion compared to patients in the lowest quartile.

Both PFA-100 CADP CT and UTXB₂ levels, when considered in combination, also significantly correlated with the frequency of VG occlusion. Figure 5 shows the frequency of VG occlusion in patients on aspirin monotherapy stratified by quartile of PFA-100 CADP CT and by upper quartile of UTXB₂. Figure 5 demonstrates that for each quartile of PFA-100 CADP CT, patients who were also in the highest quartile of UTXB₂ suffered higher rates of VG occlusion.

Example 3

Multivariate Analysis

We sought to determine the relationships of shear-dependent platelet hyper-reactivity and aspirin-insensitive thromboxane generation to other known risk factors for early VG occlusion. Prior studies have identified several patient and graft -specific variables that affect VG patency. These include the location, diameter and quality of the native target vessel as well as the diameter, number of distal anastomosis and time since implantation of the VG (10, 11, 35-41). Univariate analyses were performed on a per graft basis for the outcome of odds of occlusion using those variables deemed biologically plausible or supported by the literature in patients maintained on aspirin monotherapy. Categorical covariates were tested for collinearity using Chi squared or Fisher's exact tests, while continuous variables were similarly tested using Pearson's correlation coefficients. Highly correlated covariates, $\rho \geq 0.7$, were eliminated based upon clinical significance. Variables attaining statistical significance of $P \leq 0.15$ in the univariate analysis or those variables supported by the literature were entered into a multilevel random effects model with a random intercept for patient. Stepwise backwards elimination of variables was performed and several potential models generated. Akaike Information Criterion (AIC) was utilized for model comparison. The most parsimonious model, as determined by the AIC, was used to identify independent predictors of graft failure.

Three multivariate models were constructed that considered PFA-100 CADP CT and UTXB₂ levels as both continuous and dichotomous independent variables as well as in combination (Table 2).

Table 2. Multivariate logistic regression models for VG occlusion. Variables are ranked according to importance to model fit.

Variable	Odds of Occlusion	P value	95% CI
<u>Model 1</u>			
PFA-100 CADP CT (seconds)	0.98	0.002	0.97-0.99
Target vessel diameter ≤ 1.5 mm	2.36	0.009	1.23-4.52
ln UTXB ₂ (pg/mg creatinine)	1.73	0.05	1.01-2.99

<u>Model 2</u>			
PFA-100 CADP CT ≤ 88 sec	2.85	0.006	1.36-6.00
Target vessel size ≤ 1.5 mm	2.38	0.01	1.22-4.61
UTXB ₂ > 449 pg/mg creatinine	2.59	0.02	1.20-5.56

<u>Model 3</u>			
CADP CT ≤ 88 sec and UTXB ₂ > 449 pg/mg creatinine) versus CADP CT > 88 sec and UTXB ₂ ≤ 449 pg/mg creatinine	6.85	0.001	2.16-21.7
Target vessel size ≤ 1.5 mm	3.10	0.04	1.06-9.09

Both the PFA-100 CADP CT and UTXB₂ levels were significant independent risk factors for early VG occlusion, along with target vessel diameter ≤ 1.5 mm (historically the greatest predictor of early VG occlusion), when considered both as continuous variables (Model 1) and as binary variables based on the median value of the PFA-100 CADP CT (88 seconds) and upper quartile of UTXB₂ (449 pg/mg creatinine; Model 2). No other predictor of VG occlusion was statistically significant.

The two assays can also be used in combination to identify patients at high and low risk for VG occlusion. Patients at elevated risk include those with PFA-100 CADP CT ≤ 88 seconds and UTXB₂ > 449 pg/mg creatinine, while low risk patients would be those with PFA-100 CADP CT > 88 seconds and UTXB₂ ≤ 449 pg/mg creatinine (Model 4). The odds of VG occlusion in the high risk group was nearly 7-times that of the low risk group.

These data support the following conclusions: 1) Aspirin resistance, defined as incomplete inhibition of platelet COX-1 activity, occurs relatively infrequently in patients after CABG surgery who are taking aspirin; 2) Shear-dependent platelet hyper-reactivity and aspirin-insensitive thromboxane generation are quite common after CABG surgery; 3) Shear-dependent

platelet hyper-reactivity, as defined by a low PFA-100 CADP CT, and aspirin-insensitive thromboxane generation, defined by elevated UTXB₂ levels in patients on aspirin, are both independent predictors of VG occlusion 6 months after CABG surgery; 4) The combined use of PFA-100 CADP CT and UTXB₂ level can be used to identify patients at very high risk (i.e.~7-fold) of VG occlusion.

Example 4

Second-generation 11-dehydro-TXB₂ assay

Available assay for measuring 11-dehydro-TXB₂ include the ELISA-based assay, offered by Esoterix Inc, uses a polyclonal antibody to detect 11-dehydro-TXB₂. A second-generation ELISA-based assay that uses a monoclonal antibody to detect 11-dehydro-TXB₂ has been developed by Corgenix Medical Corporation. Because the specificities of the antibodies are different, the level of 11-dehydro-TXB₂ reported in a given sample is approximately 2.5 to 3-fold higher with the Corgenix version compared to the Esoterix version of the ELISA assay.

We repeated the 3-day and 6-month UTXB₂ determinations in available RIGOR samples using the Corgenix version of the assay and compared these results to those obtained with the first-generation Esoterix assay. Figure 6 provides the correlation between 11-dehydro-TXB₂ levels measured using the Esoterix and Corgenix assays. Figure 6 shows a high correlation between the results of the two assays. Figure 7 provides an association between VG occlusion and tertiles of UTXB₂ levels determined by the Esoterix (A) and Corgenix (B) assays in the same urine samples. Figure 7 shows the respective correlations between 6-month UTXB₂ simultaneous measurements of the two assays with VG occlusion.

Multivariate modeling was then repeated for VG occlusion substituting the 6-month UTXB₂ levels obtained using the Corgenix version of the assay for those obtained using the Esoterix version of the assay (Table 3).

Table 3. Multivariate logistic regression models for VG occlusion using UTXB₂ determined by the Corgenix assay. Variables are ranked according to importance to model fit.

Variable	Odds of Occlusion	P value	95% CI
<u>Model 1</u>			
PFA-100 CADP CT (seconds)	0.97	0.002	0.96-0.99
Target vessel diameter ≤1.5mm	2.12	0.04	1.03-4.35
Female Gender	3.03	0.01	1.26-7.29
ln UTXB ₂ (pg/mg creatinine)	1.43	0.2	0.83-2.45

Table 3. Multivariate logistic regression models for VG occlusion using UTXB₂ determined by the Corgenix assay. Variables are ranked according to importance to model fit.

Variable	Odds of Occlusion	P value	95% CI
<u>Model 2</u>			
PFA-100 CADP CT $\leq$ 88 sec	3.19	0.001	1.64-6.20
Target vessel size $\leq$ 1.5mm	2.24	0.01	1.22-4.14
UTXB ₂ >681 pg/mg creatinine	1.91	0.05	1.00-3.67
<u>Model 3</u>			
CADP CT $\leq$ 88 sec and UTXB ₂ >681 pg/mg creatinine) versus CADP CT >88 sec and UTXB ₂ $\leq$ 681 pg/mg creatinine	6.82	0.000	2.40-19.3
Female Gender	2.54	0.07	0.94-6.88
Target vessel size $\leq$ 1.5mm	1.61	0.3	0.88-3.69

UTXB₂ >681 pg/mg creatinine correlated with VG occlusion both independently and when used in combination with PFA-100 CADP CT.

These data suggest that the association between aspirin-insensitive thromboxane generation and VG occlusion is independent of the particular assay used to measure 11-dehydro-TXB₂.

Example 5

Blood and Urine Collection

Morning blood and urine samples were obtained from 288 fasted patients on chronic aspirin therapy a median of 189 days (interquartile range 182-202 days) after CABG surgery, immediately prior to coronary angiography. Using Bectin-Dickenson (Franklin Lakes, NJ) blood collection devices, blood was drawn into siliconized glass vacutainers containing EDTA (for complete blood counts), 3.2% (0.106 mol/L) and 3.8% (0.129 mol/L) sodium citrate (for platelet function testing and measurement of fibrinogen and vWF antigen) or plain glass vacutainers (for serum CRP determination). Platelet function analyses were performed within 1 hour of blood collection after being hand-carried to the laboratory and maintained at room temperature. Aliquots of centrifuged platelet-poor plasma, serum and urine were stored at -70°C until batch analyzed.

Platelet Function and Other Analyses

Complete blood counts, reticulocyte counts and immature platelet fraction were measured with an XE-2100 automated analyzer (Sysmex Corporation, Kobe, Japan). Platelet rich plasma was prepared from blood collected in 3.2% citrate by centrifugation at 100 rpm for 10 minutes. The platelet count was adjusted to 180,000 /mm³ by the addition of platelet-poor plasma and impedance aggregometry was performed after stimulation with 0.5 mM arachidonic acid using a Chrono-Log Model 560CA aggregometer (Chrono-Log, Havertown, PA). Platelets were considered non-responsive to arachidonic acid stimulation if aggregation was ≤ 1 ohms.

Shear-dependent platelet aggregation was performed on whole blood collected in both 3.2% and 3.8% citrate using a Platelet Function Analyzer-100® (Siemens Healthcare Diagnostics, Newark, DE) with both CEPI and CADP agonist cartridges according to the manufacturer's instructions. CTs in excess of 300 seconds were considered as non-closure and assigned a value of 300 seconds for statistical analysis. Because the coefficients of variance for CEPI and CADP CT performed on quality control samples was $\leq 10\%$, a single determination was performed on experimental samples unless the CT was ≥ 300 seconds or there was a significant discrepancy between CTs in 3.2% and 3.8% citrate. In the case of repeat determinations, the average CT was used for statistical analysis. The normal ranges used by our laboratory for CEPI and CADP CTs are 94-193 and 71-118 seconds, respectively.

Plasma fibrinogen was measured by a modified Clauss method using the Multifibren U kit (Siemens) and expressed in mg/dL. Plasma vWF antigen was measured by an immunoturbometric assay using the STA®-Liatest®VWF:Ag kit (Diagnostica Stago, Asnieres, France) and expressed as percent of normal control plasma. Both coagulation tests were performed on a Siemens BCS Coagulation Analyzer. Serum CRP was measured by high-sensitivity immunoturbometric assay (Tina-quant CRP (Latex); Roche Diagnostics, Mannheim, Germany) and expressed as mg/L. Measurement of 11-dehydro-thromboxane B2 in urine samples was performed in duplicate by Esoterix Laboratory Services, Inc. (Austin, TX) by ELISA and expressed as pg/mg of creatinine. Measurement of 8-isoprostane-prostaglandin F2 α in urine samples was performed in duplicate by Corgenix Medical Corporation (Broomfield, CO) by ELISA and expressed as pg/mg of creatinine.

Statistical Analysis

Only those subjects maintained on chronic aspirin therapy with non-responsiveness to arachidonic acid-induced platelet aggregation were included in the analysis. A logistic regression model was employed to determine odds ratios for low CEPI and CADP CTs performed in both 3.2% and 3.8% citrate. A low CEPI CT was defined as ≤ 193 seconds based on general acceptance in the literature as the lower limit of aspirin responsiveness for both 3.2% and 3.8% citrate. A low CADP CT was defined as ≤ 71 seconds based on the lower limit of the normal range in our laboratory. Normality assumptions for categorical variables were tested using quantile-quantile plots and the Shapiro-Francia test for normality. Skewed data were log-transformed; those that remained skewed after log transformation were treated dichotomously or categorically. Category values were chosen based on the reference ranges established by the Johns Hopkins Medical Laboratory. As no normal range has been established for urine 8-iso-prostaglandin F2 α the median value of 1073 pg/mg creatinine was chosen as the dichotomizing point for this variable.

Univariate logistic regression for odds of low CT was performed using clinically- and historically-relevant candidate predictors. Those variables with $P \leq 0.2$ were entered into the four initial multivariate models for prediction of odds of low closure time (CEPI and CADP using both 3.2% and 3.8% citrate). Thereafter, model fit was optimized using the Akaike Information Criteria (AIC), by testing stepwise removal of each variable. Lower AICs indicate improved goodness of fit of a statistical model. Collinearity among dependent variables was considered using chi-squared tests of independence for categorical or binary covariates, or Pearson's correlation coefficients for continuous variables. Potential relations between binary and continuous explanatory variables were examined using logistic regression. For pairs of collinear variables that were non-significant when together in the model, the variable with the greatest explanatory power per AIC was retained. Random effects logistic regression models were explored for the four outcomes, by including a random intercept for surgeon or hospital or both.

All statistical analysis was performed using Stata/MP 10.0 for Macintosh (StataCorp, College Station, TX, USA). Unless otherwise specified, all tests were 2-sided with significance set at $\alpha = 0.05$.

Results

Of the 288 patients on chronic aspirin therapy in the study cohort, 285 (99%) were non-responsive to arachidonic acid-induced platelet aggregation and thus included in the analysis. Subject demographics, medication profiles and major comorbidities are shown in Table 4.

5

Table 4 Patient baseline characteristics (n = 285).

<u>Characteristic</u>	<u>Number</u>
Gender	
Men	225 (79%)
Women	60 (21%)
Age, years (mean $\pm$ SD)	63.1 $\pm$ 10.0
Body Mass Index, kg/m ² (mean $\pm$ SD)	29.8 $\pm$ 6.2
Race	
White	247 (87%)
African American	28 (10%)
Asian American	8 (3%)
Native American	2 (1%)
Ethnicity	
Hispanic	8 (3%)
Non-Hispanic	277 (97%)
Diabetes	100 (35%)
Hypertension	51 (18%)
Dyslipidemia	240 (84%)
Tobacco Use	69 (24%)
Myocardial Infarction	117 (41%)
Cerebral Vascular Disease	23 (8%)
Aspirin dose	
81 mg	28 (10%)
325 mg	257 (90%)
Clopidogrel	32 (11%)
Warfarin	14 (5%)

Figure 8A shows the distribution of CEPI and CADP CTs for blood collected in 3.2% and 3.8% citrate. CEPI and CADP CTs were lower by an average of 44.9 and 10.3 seconds, respectively, in blood collected in 3.2% compared to 3.8% citrate, resulting in significantly higher percentages of patients with defined low CTs (≤ 193 for CEPI and ≤ 71 seconds for CADP cartridges; Figure 8B). The percentage of patients with both a low CEPI and CADP CT was significantly higher when measured in blood collected in 3.2% compared to 3.8% citrate (19.2% versus 2.8%, respectively, $P < 0.0001$; Figure 8C). Table 5 shows the degree of concordance between low CEPI and CADP CTs stratified by citrate concentration.

Table 5 Concordance of low CTs by cartridge and citrate concentration

	CEPI CT ≤ 193 seconds 3.2% citrate	CEPI CT > 193 seconds 3.2% citrate
CEPI CT ≤ 193 seconds 3.8% citrate	32	6
CEPI CT > 193 seconds 3.8% citrate	79	163

	CADP CT ≤ 71 seconds 3.2% citrate	CADP CT > 71 seconds 3.2% citrate
CADP CT ≤ 71 seconds 3.8% citrate	35	15
CADP CT > 71 seconds 3.8% citrate	55	178

Although average CTs were higher with 3.8% citrate, there were 21 instances (6 with CADP and 15 with CEPI cartridges) where patients with low CTs in 3.8% citrate did not have low CTs in 3.2% citrate. The correlation coefficients for low CTs between citrate concentrations were 0.39 and 0.57 for CEPI and CADP agonist cartridges, respectively.

Univariate logistic regression was performed to predict odds of low CEPI and CADP CTs in both 3.2% and 3.8% citrate (Table 6).

Table 6 Univariate analysis of potential factors associated with low CEPI and CADP CT.

	CEPI				CADP			
	3.2% Citrate		3.8% Citrate		3.2% Citrate		3.8% Citrate	
	<u>Odds Ratio</u>	<u>P-value</u>	<u>Odds Ratio</u>	<u>P-value</u>	<u>Odds Ratio</u>	<u>P-value</u>	<u>Odds Ratio</u>	<u>P-value</u>
Gender (women vs. men)	0.68	<i>0.207</i>	1.00	0.993	0.66	<i>0.212</i>	1.85	<i>0.080</i>
Age (years)	1.00	0.693	1.00	0.933	1.00	0.742	0.98	0.257
Race (white vs. non-white)	1.56	0.250	1.29	0.651	3.49	<i>0.012</i>	1.96	0.226
Ethnicity (hispanic vs. non-hispanic)	0.92	0.913	2.19	0.347	0.30	0.264	0.66	0.702
Aspirin dose (325 mg vs. 81 mg)	1.00	0.993	<i>0.53</i>	<i>0.203</i>	<i>0.42</i>	<i>0.032</i>	0.61	0.283
Clopidogrel use	1.22	0.602	1.96	<i>0.151</i>	1.33	0.463	1.66	0.252
Warfarin use	<i>1.16</i>	0.784	1.07	0.932	<i>4.20</i>	<i>0.012</i>	2.78	<i>0.079</i>
WBC (≤ 4.5 vs. $4.5\text{--}1110^3/\text{mm}^3$)*	<i>0.47</i>	<i>0.060</i>	0.55	0.337	<i>0.56</i>	<i>0.173</i>	<i>0.23</i>	<i>0.052</i>
WBC (> 11 vs. $4.5\text{--}1110^3/\text{mm}^3$)*	3.63	<i>0.128</i>	2.47	0.291	1.53	<i>0.585</i>	<i>0.68</i>	<i>0.728</i>
Hematocrit (%)	<i>1.10</i>	<i>0.009</i>	1.03	0.567	<i>1.11</i>	<i>0.007</i>	1.04	0.408
MCV (< 80 vs. $80\text{--}100$ fL)	<i>0.40</i>	<i>0.164</i>	1.08	0.921	<i>0.16</i>	<i>0.077</i>	0.79	0.767
MCV (> 100 vs. $80\text{--}100$ fL)	0.36	0.370	1.62	0.670	1.36	0.736	3.17	0.213
RDW ($> 14.5\%$ vs. $\leq 14.5\%$)	<i>0.72</i>	<i>0.199</i>	1.56	0.207	0.96	0.887	1.09	0.795
Reticulocyte count (natural log-transformed %)	0.86	0.701	1.10	0.858	1.15	0.728	1.41	0.486
Platelet Count (≥ 150 vs. $< 150 \times 10^3/\text{mm}^3$)	<i>1.58</i>	<i>0.140</i>	1.93	<i>0.192</i>	1.55	<i>0.181</i>	2.20	<i>0.088</i>
MPV (natural log-transformed fL)	<i>14.30</i>	<i>0.080</i>	5.65	0.414	6.04	0.254	0.11	0.256
IPF (natural log-transformed %)	<i>1.48</i>	<i>0.118</i>	1.22	0.580	1.02	0.938	0.82	0.514
Blood group (O vs. non-O)	<i>0.67</i>	<i>0.106</i>	0.71	0.345	<i>0.53</i>	<i>0.016</i>	<i>0.49</i>	<i>0.032</i>
Rh factor (positive vs. negative)	1.06	0.872	1.65	0.428	0.84	0.641	0.66	0.342
CRP (< 5 vs. ≥ 5 mg/L)	0.84	0.551	1.16	0.771	1.34	0.314	1.14	0.719
Fibrinogen (> 450 vs. ≤ 450 mg/dL)	1.24	0.404	0.88	0.741	<i>1.58</i>	<i>0.086</i>	1.31	0.405
vWF antigen (> 150 vs. $\leq 150\%$)	<i>1.85</i>	<i>0.014</i>	<i>4.14</i>	<i>0.001</i>	<i>3.11</i>	<i>0.000</i>	<i>3.70</i>	<i>0.000</i>
Urine 11-dehydro-TXB ₂ (≥ 400 vs. < 400 pg/mg creatinine)	<i>2.05</i>	<i>0.005</i>	<i>1.77</i>	<i>0.108</i>	1.55	<i>0.099</i>	1.27	0.465
Urine 8-iso-PGF _{2α} (≥ 1072 vs. < 1072 pg/mg creatinine)	<i>1.37</i>	0.228	1.18	0.678	<i>1.64</i>	<i>0.076</i>	1.57	<i>0.184</i>

Variables included in subsequent multivariate models are italicized.

WBC = white blood cell count; MCV = mean corpuscular volume; RDW = red cell distribution width; MPV = mean platelet volume; IPF = immature platelet fraction; vWF = von Willebrand factor; 11-dehydro-TXB₂ = 11-dehydro-thromboxane B₂; 8-iso-PGF_{2 α} = 8-isoprostane-prostaglandin F_{2 α} .

An elevated vWF antigen level > 150% (the upper limit of normal in our laboratory) was the only significant predictor of a low CT consistently identified in all four combinations of agonist cartridge and citrate concentration. Multivariate analysis identified several factors that differentially correlated with odds of low CADP and CEPI CTs depending on whether the blood was collected in 3.2% vs. 3.8% citrate (Tables 7 and 8).

Table 7 Multivariate analysis of factors associated with low CEPI CT*.

	3.2% Citrate		3.8% Citrate	
	<u>Odds Ratio</u>	<u>P-value</u>	<u>Odds Ratio</u>	<u>P-value</u>
vWF antigen (> 150 vs. ≤ 150%)	NS	NS	4.14	0.000
Blood group (O vs. non-O)	0.53	0.030	NS	NS
Hematocrit (%)	1.10	0.036	NS	NS
IPF (natural log-transformed %)	1.68	0.064	NS	NS
Urine 11-dehydro-TXB ₂ (≥ 400 vs. <400 pg/mg creatinine)	1.66	0.093	NS	NS

* Shown in descending order of strength of association assessed by t-statistic and importance to model fit.

NS = not significant in univariate analysis and/or not included in the optimized model;
vWF = von Willebrand factor; IPF = immature platelet fraction; 11-dehydro-TXB₂ = 11-dehydro-thromboxane B₂.

Table 8 Multivariate analysis of factors associated with low CADP CT*.

	3.2% Citrate		3.8% Citrate	
	<u>Odds Ratio</u>	<u>P-value</u>	<u>Odds Ratio</u>	<u>P-value</u>
vWF antigen (> 150 vs. ≤ 150%)	2.99	0.001	2.56	0.016
Race (white vs. non-white)	3.69	0.019	NS	NS
Warfarin use	6.11	0.028	2.90	0.103
Hematocrit (%)	1.11	0.029	NS	NS
Urine 8-iso-PGF _{2α} (≥ 1072 vs. <1072 pg/mg creatinine)	1.79	0.062	NS	NS
Platelet Count (≥ 150 vs. <150 × 10 ³ mm ³)	2.03	0.065	NS	NS
Gender (women vs. men)	0.52	0.137	NS	NS
Blood group (O vs. non-O)	NS	NS	0.55	0.108

* Shown in descending order of strength of association assessed by t-statistic and importance to model fit.

NS = not significant in univariate analysis and/or not included in the optimized model;

vWF = von Willebrand factor; 8-iso-PGF_{2α} = 8-isoprostane-prostaglandin F_{2α}.

For both agonist cartridges, collection of blood in 3.8% rather than 3.2% citrate was associated with fewer variables correlating with low CT. A vWF antigen level > 150% was the only significant predictor of low CEPI and CADP CT in blood samples collected in 3.8% citrate.

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We Claim:

1. A method for identifying an individual at elevated risk for an adverse cardiovascular event after coronary or peripheral arterial bypass graft surgery comprising: conducting a platelet function analysis on a sample of blood from an individual to determine the degree of platelet reactivity, wherein if the degree of platelet reactivity is elevated, the individual is at elevated risk of the adverse cardiovascular event.
2. The method of claim 1, wherein the adverse cardiovascular event comprises thrombosis.
3. The method of claim 1, wherein the adverse cardiovascular event comprises vein graft occlusion.
4. The method of claim 1, wherein the method is conducted prior to coronary arterial or peripheral arterial bypass graft surgery.
5. The method of claim 1, wherein the method is conducted minutes, hours or one, two, three, four, five or six day(s), one week, two weeks, three weeks, four weeks, or a month prior to prior to coronary arterial or peripheral arterial bypass graft surgery.
6. The method of claim 1, wherein the method is conducted after coronary arterial or peripheral arterial bypass graft surgery.
7. The method of claim 1, wherein the method is conducted at least four, five or six days, or one, two, three or four weeks, or one, two, three, four, five, six, seven, eight, nine, ten, eleven or twelve month(s) after coronary arterial or peripheral arterial bypass graft surgery.
8. The method of claim 1, wherein the method is conducted at least monthly or yearly post coronary arterial or peripheral arterial bypass graft surgery.
9. The method of claim 1, wherein the blood sample from the individual is whole blood or platelet-rich plasma.
10. The method of claim 1, wherein the platelet reactivity comprises the shear-dependent platelet reactivity.
11. The method of claim 10, wherein the platelet reactivity is measured using a Platelet Function Analyzer.
12. The method of claim 11, wherein the Analyzer is a Platelet Function Analyzer-100 assay.
13. The method of claim 11, wherein the Analyzer uses a collagen/ADP agonist cartridge.

14. The method of claim 11, wherein platelet reactivity is measured using closure time.

15. The method of claim 14, wherein an elevated degree of platelet reactivity is indicated by lower closure time.

16. The method of claim 15, wherein a lower closure time is equal to or below 150, 125, 100, 90, 88, 74, 73, 72 or 71 seconds.

17. The method of claim 11, wherein a high closure time indicates a reduced degree of platelet reactivity and a reduced risk of an adverse cardiovascular event.

18. The method of claim 17, wherein a high closure time is above 168, 170, 180, 190, 200, 250, 300 seconds.

19. The method of claim 11, wherein the closure time rate is proportional to the risk rate, with progressively reduced closure times associated with progressively increasing risk of an adverse cardiovascular event.

20. The method of claim 19, wherein individuals having a lower closure time rate of equal to or less than 88 seconds has a more than 2.5-fold higher frequency of having cardiovascular disease or vein graft occlusion than individuals with closure time rate of more than 88 seconds.

21. A kit for identifying an individual at elevated risk for an adverse cardiovascular event after coronary or peripheral arterial bypass graft surgery, said kit containing: a) reagents for conducting a platelet function analysis to determine platelet reactivity, and optionally b) a script or instructions for determining whether the individual is at elevated risk for adverse cardiovascular events.

22. Use of an assay comprising platelet function analysis to identify individuals at elevated risk for adverse cardiovascular events prior to or after coronary artery bypass surgery, comprising: conducting a platelet function analysis on a sample of blood from an individual to determine the degree of platelet reactivity, wherein if the degree of platelet reactivity is elevated, then the individual is at elevated risk of the adverse cardiovascular event.

FIG. 1

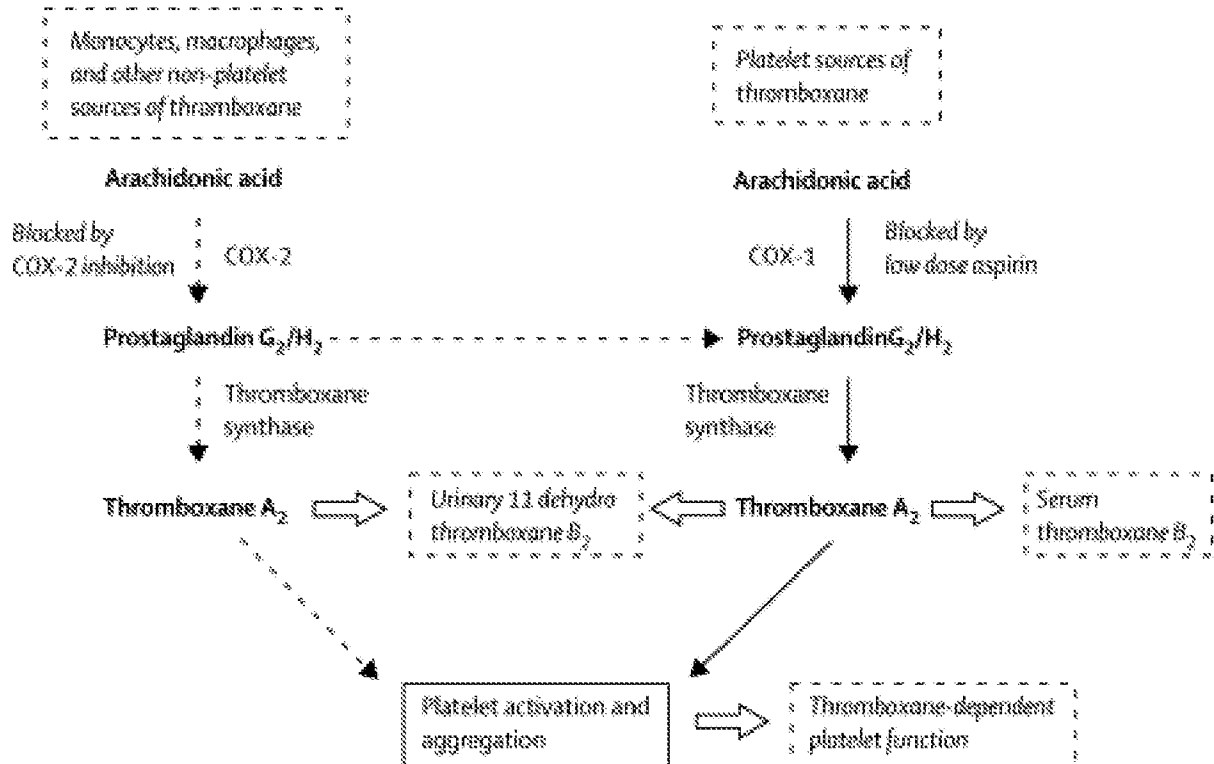


FIG. 2

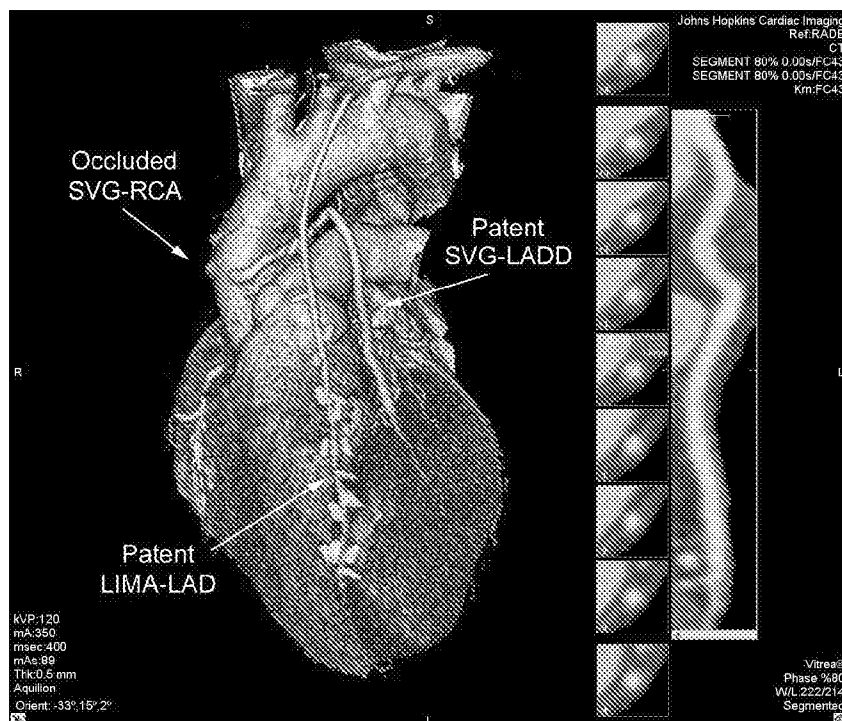


FIG. 3

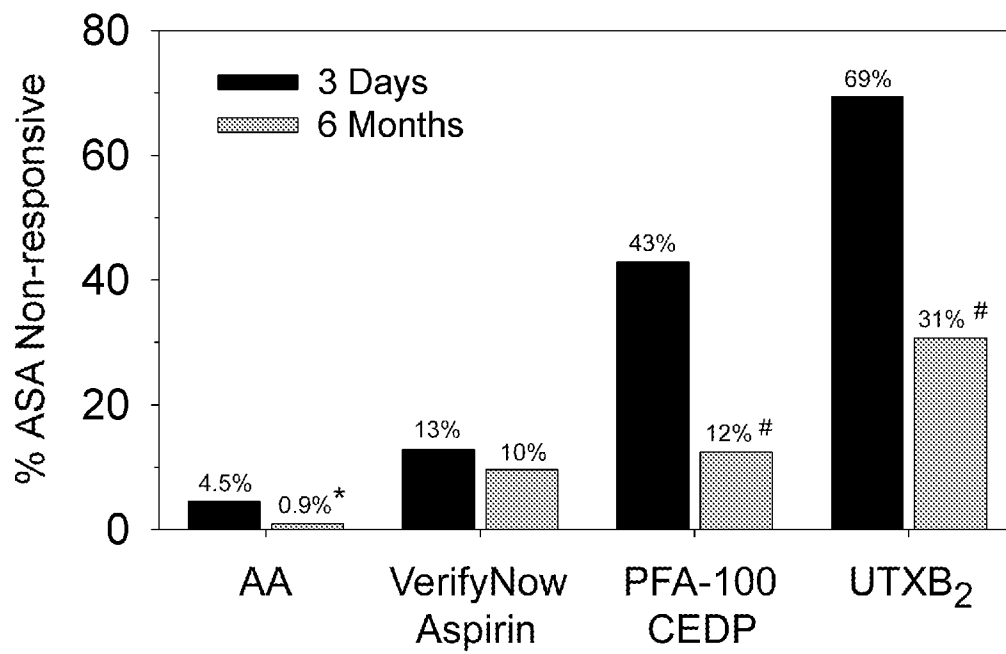
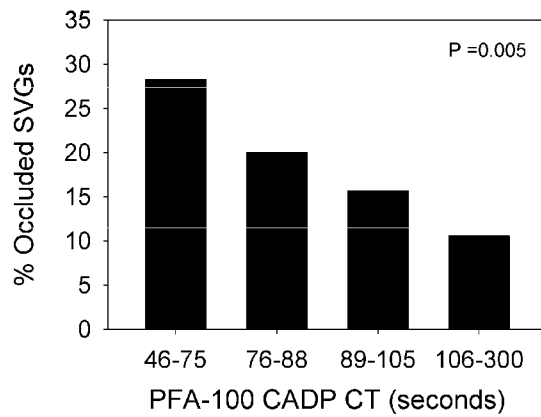


FIG. 4

A.



B.

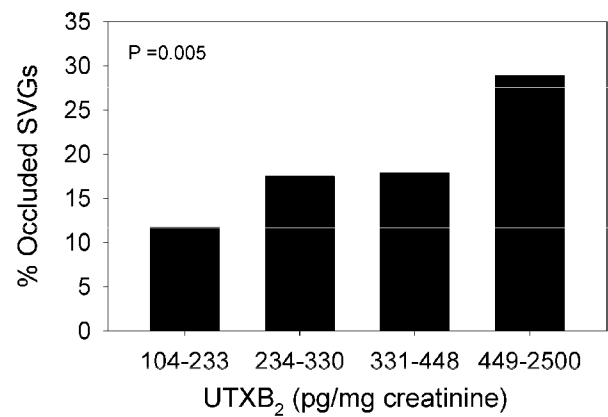


FIG. 5

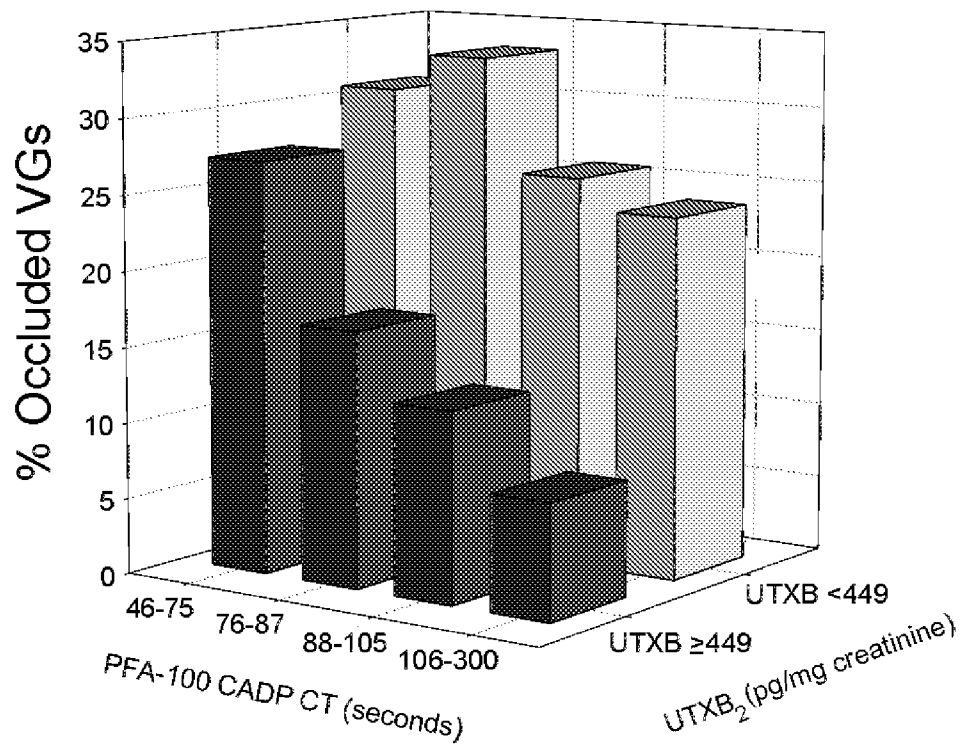


FIG. 6

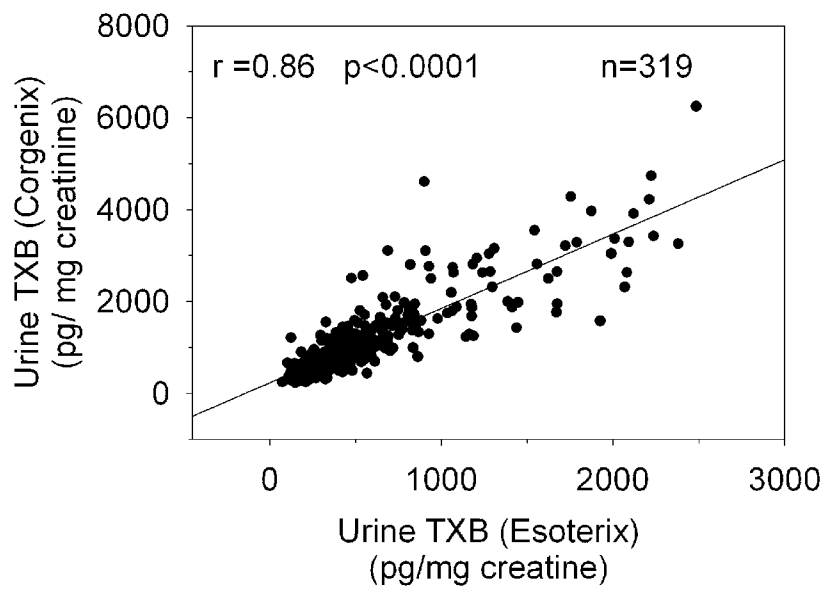


FIG. 7

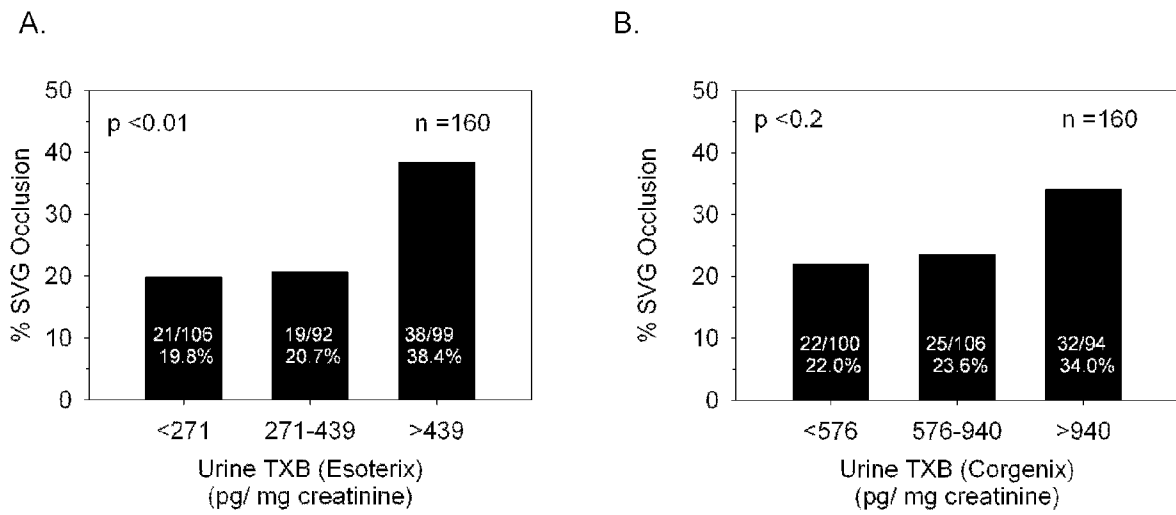
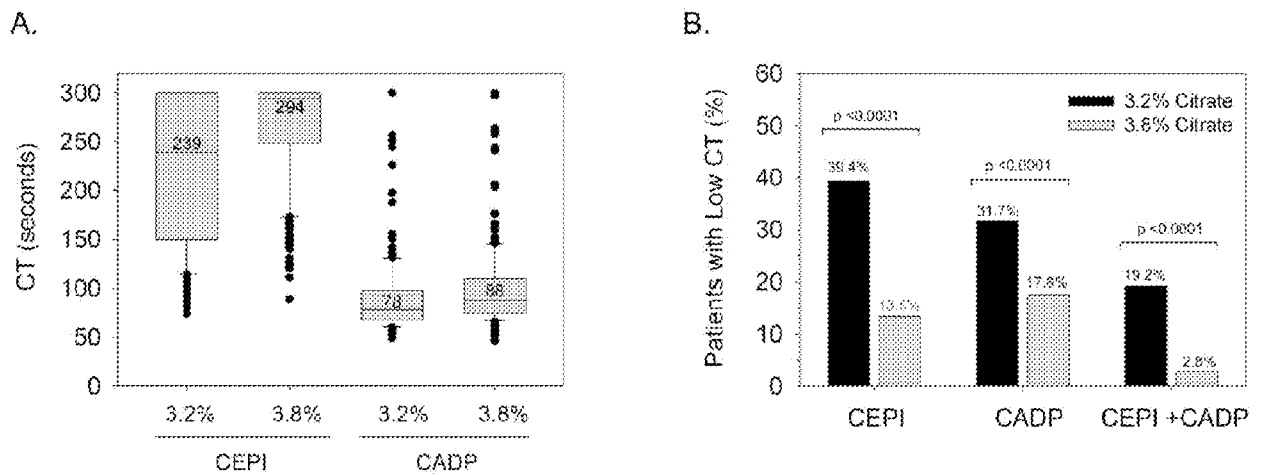


FIG. 8



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 09/59275

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12Q 1/56 (2009.01)

USPC - 435/13

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

USPC - 435/13

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 435/13 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PubWEST (USPT,PGPB,EPAB,JPAB); Google

Search Terms Used: cardiovascular, platelet function analysis, coronary, peripheral arterial, bypass graft surgery, vein graft occlusion, platelet function analysis, thrombosis, risk, assay

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2006/0199239 A1 (Gurbel) 07 September 2006 (07.09.2006), abstract, para [0020],[0029], [0039], [0096], [0114], [0128], [0162], [0169], [0173], [0181], [0211]	1-12, 21-22 ----- 13-20
Y	US 2008/0009024 A1 (Christie) 10 January 2008 (10.01.2008), para [0030], [0037], [0043], [0047]	13-20
Y	US 2006/0269978 A1 (Haworth et al.) 30 November 2006 (30.11.2006), para [0140]	18

☐ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

22 November 2009 (22.11.2009)

Date of mailing of the international search report

04 DEC 2009

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