Thromboxane A$_2$ Generation, in the Absence of Platelet COX-1 Activity, in Patients With and Without Atherothrombotic Myocardial Infarction

Andrew P. DeFilippis, MD; Oluwasegun S. Oloyede, MD; Efstathia Andrikopoulou, MD; Amy K. Saenger, PhD; Joel M. Palachuvattil; Yetunde A. Fasoro; Eliseo Guallar, MD; Roger S. Blumenthal, MD; Thomas S. Kickler, MD; Allan S. Jaffe, MD; Gary Gerstenblith, MD; Steven P. Schulman, MD; Jeffrey J. Rade, MD

**Background:** Aspirin’s therapeutic action is via inhibition of platelet cyclooxygenase 1 (COX-1) thromboxane A$_2$ (TxA$_2$) production. The aim of this study was to evaluate TxA$_2$ production, in the absence of platelet COX-1 activity, in coronary atherosclerotic heart disease patients with and without atherothrombotic myocardial infarction (MI).

**Methods and Results:** TxA$_2$ production, in the absence of platelet COX-1 activity, was evaluated in 44 patients taking aspirin on 3 commercially available assays that detect metabolites of TxA$_2$ in the urine. Two assays measure urine 11-dehydro-thromboxane B$_2$ (TxB$_2$) alone and 1 measures urine 11-dehydro-TxB$_2$ plus 11-dehydro-2,3-dinor-TxB$_2$. Platelet COX-1 inhibition was confirmed on <10% platelet aggregation in response to ≥1 mmol/L arachidonic acid. Median urine 11-dehydro-TxB$_2$ was no different in those with and without a diagnosis of atherothrombotic MI (325 vs. 311 pg/mg creatinine, P=0.59 via polyclonal ELISA) and (312 vs. 244 pg/mg creatinine, P=0.11 via LC-MS/MS). Median urine 11-dehydro-TxB$_2$ plus 11-dehydro-2,3-dinor-TxB$_2$, however, was higher in those with vs. those without a diagnosis of atherothrombotic MI (1,035 vs. 606 pg/mg creatinine, P=0.03 via monoclonal ELISA).

**Conclusions:** Differences in TxA$_2$ production, in the absence of platelet COX-1 activity, between those with vs. without atherothrombotic MI were not observed when TxA$_2$ generation was assessed on 11-dehydro-TxB$_2$ production alone (polyclonal ELISA or LC-MS/MS), but differences were observed when TxA$_2$ generation was assessed using 11-dehydro-TxB$_2$ plus 11-dehydro-2,3-dinor-TxB$_2$ (monoclonal ELISA). These findings highlight important differences between different commercially available assays for TxA$_2$ generation and suggest that 11-dehydro-2,3-dinor-TxB$_2$ may be critical to the biology of atherothrombosis.  (*Circ J* 2013; **77:** 2786–2792)

**Key Words:** Aspirin resistance; Atherothrombosis; Thromboxane
yet many patients still experience atherothrombotic events despite adequate aspirin therapy. Patients who produce TxA₂ despite aspirin therapy have been labeled “aspirin-resistant”. Prior work by our group and others, however, has demonstrated that significant production of TxA₂ occurs despite the complete suppression of platelet COX-1 activity. In patients with stable coronary artery disease (CAD) treated with adequate doses of aspirin, continued thromboxane production, as evidenced by elevated 11-dehydro-TxB₂ concentration, has been correlated with the occurrence of future cardiovascular events. The detection of TxA₂ production, in the absence of platelet COX-1 activity (on ELISA), has led to a recent consensus decision to limit the definition of aspirin resistance to the failure of aspirin to inhibit platelet COX-1 activity, a rare genetic defect.

In the present study, we evaluated TxA₂ production, in the absence of platelet COX-1 activity, in coronary atherosclerotic heart disease patients with and without atherothrombotic MI. All participants underwent coronary angiography as well as arachidonic acid aggreometry to establish the efficacy of aspirin therapy (complete suppression of platelet COX-1 activity). We measured TxA₂ production using 3 different assays that measure metabolites of TxA₂: in the urine: a monoclonal antibody ELISA (Caymen Chemical, MI, USA); a polyclonal antibody ELISA (Caymen Chemical); and a liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay developed at the Mayo Clinic. All 3 assays measure 11-dehydro-TxB₂, a stable metabolite of TxA₂, but subsequent to the design of this study it was discovered that the monoclonal antibody ELISA used in this study measures both 11-dehydro-TxB₂ and 11-dehydro-2,3-dinor-TxB₂. We had hypothesized that TxA₂ production, in the absence of platelet COX-1 activity, would be greater in those with vs. without atherothrombotic MI.

**Methods**

**Subjects**

Participants were recruited from a single academic medical center between September 2008 and August 2010. Those enrolled met the following criteria: (1) ≥18 years of age; (2) scheduled for coronary angiography within 48h; and (3) reported ingesting an adequate aspirin dose (2162mg on the prior calendar day but within the last 24h of presentation, or ≥81mg/day for the last 7 days). Subjects with a platelet count <100,000/mm³ or who were unable to provide a urine sample were excluded.

Two general categories of patients were sought for enrollment prior to coronary angiography: (1) ACS subjects, including those with ST- or non-ST-elevation MI, as confirmed by electrocardiographic (ECC), and biochemical criteria (Table S1); and (2) non-ACS subjects, excluding those with vascular events or interventions during the past 12 weeks, coronary bypass surgery in the prior year, or findings consistent with unstable angina (Table S1). ACS and non-ACS enrollment criteria were based on criteria developed for the Acute Catheterization and Urgent Intervention Triage strategY (ACUITY) trial and The Atlantic Cardiovascular Patient Outcomes Research Team (C-PORT) Trial (Table S1). The study was approved by the Johns Hopkins Institutional Review Board (IRB). All patients provided written informed consent.

**History, Physical Exam, and ECG Data**

A single study physician interviewed all participants and finalized history, physical exam, and ECG data prior to any measurement of TxB₂. The study interview included specific questioning with regard to the timing, dose, and frequency of aspirin and non-steroidal anti-inflammatory drug (NSAID) use in the 7 days prior to enrollment and the nature and timing of angina symptoms. The medical record was used to aid in collection of pertinent medical history (ie, timing of medication). Physical exam data were ascertained by the study physician from the medical record at the time of enrollment. All ECGs were read by the study physician in accordance with a priori study guidelines. Standard laboratory data (troponin, creatinine, blood cell and platelet counts) were obtained from the medical record.

**Biochemical Data**

A urine specimen was collected from each participant at the time of enrollment, just prior to cardiac catheterization, and specimens were centrifuged, aliquotted, and stored at ~70°C until analyzed. Metabolites of TxA₂ were quantified on 3 urine assays. First, a monoclonal antibody ELISA (AspirinWorks Test Kit, catalog no.: 10010153; Caymen Chemical) was performed by Esoterix Laboratory Services, a Clinical Laboratory Improvement Amendments (CLIA)-approved laboratory facility. This assay is FDA-approved for the measurement of urine 11-dehydro-TxB₂, but significant cross-reactivity with 11-dehydro-2,3-dinor-TxB₂ has recently been established. Therefore, measurements from this assay represent 11-dehydro-TxB₂ plus 11-dehydro-2,3-dinor-TxB₂. The ranges of the assay’s inter-assay and intra-assay coefficients of variation were 5–8% and 7–14%, respectively. The reportable range for this monoclonal ELISA test is 300–4,000 pg/ml. Values below (n=7) or above (n=6) these limits were imputed as 200 and 5,000 pg/ml, respectively. Second, a polyclonal antibody ELISA (catalog no.: 519501; Caymen Chemical) was also performed by Esoterix Laboratory Services. This assay has a reported cross-reactivity with the TxA₂ metabolite, 11-dehydro-2,3-dinor-TxB₂ of 11.4%. The methods used for assessing 11-dehydro-TxB₂ via the polyclonal ELISA in this study were identical to those used in large-outcome trials that demonstrated inter-assay and intra-assay coefficients of variation of 9.8% and 12.1%, respectively, at 31.3 pg/ml of 11-dehydro-TxB₂. And third, a quantitative analysis of urine TxB₂ was conducted using a novel LC-MS/MS assay at the Mayo Clinic, Mayo Medical Laboratories, Rochester, Minnesota, USA. Urine samples were adjusted to a pH of 2.0±0.2 and incubated for 3h at ambient temperature to force all urinary thromboxane into the closed ring form that is recognized by the LC-MS/MS method. After addition of d4 internal standard (d4-11-d TxB₂) to each sample, the samples were positive pressure-filtered, and the 11-d TxB₂ was separated from the urine matrix via turboflow online extraction. A Cyclone MAX anion-exchange column (Thermo Fisher Scientific, Franklin, MA, USA) was used for the extraction, and a Waters Xbridge C8 (Waters, Milford, MA, USA) was used for separation from other prostaglandins. From this column, the samples were transferred to an API 5000 MS/MS for instrumental analysis. Intra-assay precision was 4.4% and 2.9% for low (412 pg/ml)- and high (2,826 pg/ml)-level urine pools, respectively. Inter-assay precision was 9.8% and 11.9% for low (624 pg/ml) and high (4,782 pg/ml)-level urine pools, respectively.

All measurements were performed in duplicate, and the average 11-dehydro-TxB₂ or 11-dehydro-TxB₂ plus 11-dehydro-2,3-dinor-TxB₂: result is reported relative to urinary creatinine concentration. All samples were assessed on each of the 3 assays in 1 or 2 batches. The monoclonal antibody ELISA assay defines an abnormal thromboxane level as a “TxB₂” level >1,500 pg/ml creatinine, and measures above this level in subjects on aspirin therapy “indicate a lack of aspirin effect” Cut-offs for elevated thromboxane have not been established for the polyclonal antibody ELISA or LC-MS/MS methods.
Serum troponin I concentration was assessed with the Beckman Coulter troponin I assay in the CLIA-approved Johns Hopkins Clinical Laboratory. For this assay, the 99% cut-off level for a healthy population is 0.04 ng/ml, but the coefficient of variance does not fall below 10% until the level reaches 0.06 ng/ml, which was used as the cut-off point to define a positive test at Johns Hopkins and thus in this study.

**Assessment of Platelet COX-1 Activity**

A blood sample was obtained at the time of urine collection for assessment of platelet COX-1 activity. Platelet COX-1 activity in response to 1 mmol/L arachidonic acid was measured on light transmission aggregometry using a Chrono-Log Model 700 aggregometer in accordance with established methodology. Changes in absorbance were converted to percent aggregation by reference to the absorbances of platelet-rich plasma and platelet-poor plasma. A positive test for aggregation, indicating incomplete inhibition of COX-1, was defined as a >10% increase in light transmission in response to 1 mmol/L arachidonic acid. To ensure reagent potency and validity of the aggregometry methodology, control samples not exposed to any aspirin, NSAID, or anti-platelet drug for the previous 7 days were run using the same reagents and methodology within 24 h of study subject testing; most control subjects were volunteers from the aggregation laboratory. For the assay to be considered valid, control samples had to be >80% aggregated in response to <1 mmol/L arachidonic acid.

**Coronary Angiography**

All coronary angiography images were digitally recorded and independently reviewed by a single board-certified cardiologist blinded to all study biomarkers and patient clinical data. The reader rated the degree of luminal stenosis and the presence of thrombi as present, absent, or equivocal according to the Zack reader rated the degree of luminal stenosis and the presence of thrombi as present, absent, or equivocal according to the Zack methodology, control samples not exposed to any aspirin, NSAID, or anti-platelet drug for the previous 7 days were run using the same reagents and methodology within 24 h of study subject testing; most control subjects were volunteers from the aggregation laboratory. For the assay to be considered valid, control samples had to be >80% aggregated in response to <1 mmol/L arachidonic acid.

**Coronary Angiography**

All coronary angiography images were digitally recorded and independently reviewed by a single board-certified cardiologist blinded to all study biomarkers and patient clinical data. The reader rated the degree of luminal stenosis and the presence of thrombi as present, absent, or equivocal according to the Zack criteria in all major coronary vessels.

**Clinical Diagnosis of Atherothrombotic MI**

At the time of hospital discharge (post-cardiac catheterization), the clinical attending cardiologist was presented with a summary of the Joint ESC/ACCF/AHA/WHF universal definition for acute MI and the classification of causes (including type 1, ie, atherothrombotic). The cardiologist was then asked to determine whether each subject’s most likely diagnosis was an acute MI consistent with the Joint ESC/ACCF/AHA/WHF criteria and to classify acute MIs in accordance with the Joint ESC/ACCF/AHA/WHF Task Force guidelines. The clinical attending cardiologist had access to all clinical data, including coronary angiography and conventional myocardial necrosis biomarkers (troponin I, CK, and CK-MB) but did not have access to the TxB2 results. Subjects were further stratified to more objectively define study phenotypes (study atherothrombotic MI, study non-atherothrombotic MI, and study stable CAD) using a combination of historical, biochemical, clinical, and angiographic criteria (Table 1).

**Statistical Analysis**

Correlations among the 3 assays designed to assess TxA2 production were assessed using Spearman correlation coefficients. Differences in the measure of TxA2 metabolites between the 3 assays were assessed and differences between the monoclonal antibody ELISA and the polyclonal antibody ELISA/LC-MS/MS were attributed to the detection of 11-dehydro-2,3-dinor-TxB2 in addition to 11-dehydro-TxB2. Median 11-dehydro-TxB2 concentration (via the polyclonal antibody ELISA and LC-MS/MS) between subjects with and without a World Health Federation (WHF) diagnosis of type 1 atherothrombotic MI, as defined in the previous section, were compared using the Wilcoxon rank-sum test. Median 11-dehydro-TxB2 concentration between patients with and without a WHF diagnosis of type 1 atherothrombotic MI, as defined in the previous section, were compared using the Wilcoxon rank-sum test. Median 11-dehydro-TxB2: 11-dehydro-2,3-dinor-TxB2 level was also compared between subjects with the study definition of atherothrombotic MI and those with the study definition of non-atherothrombotic CAD (type 2 and stable CAD, Table 1) via the Wilcoxon rank-sum test. Preliminary estimates were used to ensure adequate power to detect a 25% difference in log-11-dehydro-TxB2 and 11-dehydro-TxB2: 11-dehydro-2,3-dinor-TxB2 between subjects with and those without a WHF diagnosis of type 1 atherothrombotic MI. Variables were log transformed to achieve a normal distribution, but results were transformed back to and presented on a geometric scale for ease of interpretation and to allow consistency with clinical practice.

**Results**

A total of 56 subjects were enrolled in the study. We excluded 12 subjects due to inadequate aspirin therapy, determined as >10% platelet aggregation in response to 1 mmol/L arachidonic acid. Of the remaining 44 subjects with arachidonic acid-proven adequate aspirin therapy, 39 subjects produced a urine specimen of sufficient volume to permit the measurement of TxA2 metabolites using all 3 assays. Subject characteristics are listed in Table 2.

Sixteen subjects were classified by the attending physician as having type 1 atherothrombotic MI via WHF criteria, and 27 subjects as not meeting the criteria for a type 1 atherothrombotic MI via WHF criteria (Table 2). Although both groups had evidence of ischemia according to mean peak troponin level, subjects with WHF type 1 atherothrombotic MI had a statistically significantly higher troponin level. Both groups had sig-

### Table 1. Definition of Study Phenotypes

| Study phenotype | Criteria |
|-----------------|----------|
| Study atherothrombotic MI | Peak troponin (mg/dl) >0.1, Clinical discharge diagnosis Type 1 MI, Blinded angiographic assessment Thrombus |
| Study non-atherothrombotic MI | Peak troponin (mg/dl) >0.1, Clinical discharge diagnosis Type 2 MI, Blinded angiographic assessment No thrombus |
| Study stable CAD (not ischemic) | Peak troponin (mg/dl) <0.06, Clinical discharge diagnosis No ACS, Blinded angiographic assessment >50% luminal stenosis, no thrombus |

†Joint ESC/ACCF/AHA/WHF universal definition. ACS, acute coronary syndrome; CAD, coronary artery disease; MI, myocardial infarction.
were assayed using 11-dehydro-TxB2 alone (polyclonal antibody ELISA and LC-MS/MS; Table 4). The observed power to detect a 25% difference in log 11-dehydro-TxB2 between groups with the polyclonal antibody ELISA and LC-MS/MS was 98%. Again, TxA2 metabolites, assessed using the assay that included both 11-dehydro-TxB2 plus 11-dehydro-2,3-dinor-TxB2 concentrations (via the monoclonal antibody ELISA assay) were found to be significantly higher (1,117 vs. 542 pg/mg creatinine, P=0.02; Figure B; Table 4) in those who met the study criteria for atherothrombotic MI (n=6) than in those who did not (ie, who were considered for this analysis to have non-atherothrombotic MI and stable CAD, n=12).

The median (25th–75th percentiles) concentration of 11-dehydro-TxB2 plus 11-dehydro-2,3-dinor-TxB2 for the entire study cohort was 702 pg/mg creatinine (513–1,250 pg/mg creatinine; monoclonal antibody ELISA). Median (25th–75th percentiles) concentration for the measure of 11-dehydro-TxB2 alone was significant but similar levels of cardiovascular risk factors, and CAD identified on coronary angiography. No statistically significant differences were observed between these groups when TxA2 metabolites were assessed on measurement of 11-dehydro-TxB2 alone (polyclonal antibody ELISA or LC-MS/MS assay; Table 3). The observed power to detect a 25% difference in log 11-dehydro-TxB2 between groups with the polyclonal antibody ELISA and LC-MS/MS assays was 99%. When TxA2 production assessment included the measure of 11-dehydro-TxB2 plus 11-dehydro-2,3-dinor-TxB2, as measured by monoclonal antibody ELISA testing, however, the level was significantly higher in WHF type 1 atherothrombotic MI subjects than in those without this diagnosis (1,035 vs. 606 pg/mg creatinine, P=0.03; Figure A; Table 3).

When subjects were stratified according to the more rigorous study criteria for atherothrombotic MI (Table 1), no statistically significant differences were observed when TxA2 metabolites were expressed using 11-dehydro-TxB2 alone (polyclonal antibody ELISA and LC-MS/MS; Table 4). The observed power to detect a 25% difference in log 11-dehydro-TxB2 between groups with the polyclonal antibody ELISA and LC-MS/MS was 98%. Again, TxA2 metabolites, assessed using the assay that included both 11-dehydro-TxB2 plus 11-dehydro-2,3-dinor-TxB2 concentrations (via the monoclonal antibody ELISA assay) were found to be significantly higher (1,117 vs. 542 pg/mg creatinine, P=0.02; Figure B; Table 4) in those who met the study criteria for atherothrombotic MI (n=6) than in those who did not (ie, who were considered for this analysis to have non-atherothrombotic MI and stable CAD, n=12).

The median (25th–75th percentiles) concentration of 11-dehydro-TxB2 plus 11-dehydro-2,3-dinor-TxB2 for the entire study cohort was 702 pg/mg creatinine (513–1,250 pg/mg creatinine; monoclonal antibody ELISA). Median (25th–75th percentiles) concentration for the measure of 11-dehydro-TxB2 alone was
The present study of cardiac patients with atherothrombotic and non-atherothrombotic coronary disease has demonstrated adequate linear correlations but significant quantitative differences in the measurement of TxA₂ production, in the absence of platelet COX-1 activity, between those with vs. those without WHF atherothrombotic MI was not observed when TxA₂ generation was assessed on 11-dehydro-TxB₂ production alone (polyclonal ELISA or LC-MS/MS assays), but differences were observed when TxA₂ generation was assessed by measurement of 11-dehydro-TxB₂ plus 11-dehydro-2,3-dinor-TxB₂ (monoclonal ELISA assay). We hypothesized that TxA₂ production, in the absence of platelet COX-1 activity, might be greater in those with WHF atherothrombotic MI because of the central role played by thromboxane in platelet activation in the pathophysiology of atherothrombosis.

The process of platelet activation, however, is multifactorial, involving dozens of known factors and, likely, dozens more yet to be discovered; all these factors together determine platelet activation, and this cooperative effect may preclude any 1 factor from determining platelet activation.

While other studies reported differences in TxA₂ production between those with and without ACS, those studies were limited by inconsistent aspirin use, a lack of biochemical assessment of aspirin action (arachidonic acid aggregometry), and a lack of adequate objective assessment of coronary thrombosis via angiography. While other studies reported differences in TxA₂ production between those with and without ACS, those studies were limited by inconsistent aspirin use, a lack of biochemical assessment of aspirin action (arachidonic acid aggregometry), and a lack of adequate objective assessment of coronary thrombosis via angiography.

The present study of cardiac patients with atherothrombotic and non-atherothrombotic coronary disease has demonstrated adequate linear correlations but significant quantitative differences in the measurement of TxA₂ production, in the absence of platelet COX-1 activity, between those with vs. those without WHF atherothrombotic MI was not observed when TxA₂ generation was assessed on 11-dehydro-TxB₂ production alone (polyclonal ELISA or LC-MS/MS assays), but differences were observed when TxA₂ generation was assessed by measurement of 11-dehydro-TxB₂ plus 11-dehydro-2,3-dinor-TxB₂ (monoclonal ELISA assay). We hypothesized that TxA₂ production, in the absence of platelet COX-1 activity, might be greater in those with WHF atherothrombotic MI because of the central role played by thromboxane in platelet activation in the pathophysiology of atherothrombosis. The process of platelet activation, however, is multifactorial, involving dozens of known factors and, likely, dozens more yet to be discovered; all these factors together determine platelet activation, and this cooperative effect may preclude any 1 factor from determining platelet activation. While other studies reported differences in TxA₂ production between those with and without ACS, those studies were limited by inconsistent aspirin use, a lack of biochemical assessment of aspirin action (arachidonic acid aggregometry), and a lack of adequate objective assessment of coronary thrombosis via angiography.

**Table 4. TxA₂ Metabolite Concentration vs. Study-Defined ACS Phenotype and Assay Type**

| Assay          | Study phenotype non-atherothrombotic MI + stable CAD (Median (25th–75th percentile) (pg/mg creatinine)) | Study phenotype atherothrombotic MI (Median (25th–75th percentile) (pg/mg creatinine)) | Wilcoxon rank-sum |
|----------------|---------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------|------------------|
| Mono-ELISA     | 11-dehydro-TxB₂ plus 11-dehydro-2,3-dinor-TxB₂                                                   | 12 542 (458–917)                                                                | 1,117 (702–1905) | 0.02             |
| LC-MS/MS       | 11-dehydro-TxB₂                                                                                    | 13 194 (106–325)                                                                | 350 (222–473)    | 0.1              |
| Poly-ELISA     | 11-dehydro-TxB₂                                                                                    | 11 317 (165–468)                                                                | 299 (237–414)    | 0.78             |

†Sufficient samples not available for every subject for each assay. Abbreviations as in Tables 1, 3.
of platelet COX-1 activity, as assessed by 3 commercially available assays. Urine 11-dehydro-TxB2 is thought to be the most accurate biomarker of endogenous TxA2 production because plasma measures are confounded by in vitro release of TxA2/TxB2 during phlebotomy, and by the fact that 11-dehydro-2,3-dinor-TxB2: excretion into the urine is non-linear. Although all 3 assays report measuring 11-dehydro-TxB2, the median “11-dehydro-TxB2” concentration for the monoclonal ELISA assay was 226% higher than that measured on the polyclonal antibody ELISA and 275% higher than that measured on LC-MS/MS. Significant differences in quantitative analysis between the monoclonal antibody ELISA and the other 2 assays, particularly the LC-MS/MS, which is a technology that adequately controls for cross-reactivity, suggest that the monoclonal antibody ELISA method is detecting an additional analyte. Analysis of samples from patients undergoing coronary artery bypass grafting on aspirin therapy in the RIGOR trial has recently shown that the monoclonal antibody ELISA differs from the polyclonal ELISA because of significant cross-reactivity with 11-dehydro-2,3-dinor-TxB2. This finding is consistent with the differences seen between the assays used in the present cohort of coronary atherosclerotic heart disease patients. Furthermore, in the RIGOR study, although a linear relationship was observed between the measurements of “11-dehydro-TxB2” in the 2 assays (measuring 11-dehydro-TxB2 alone and the other measuring 11-dehydro-TxB2 plus 11-dehydro-2,3-dinor-TxB2), as seen in the present study, the mean bias between the 2 assays was 35% on Bland-Altman analysis. This finding suggests that the monoclonal ELISA (which measures 11-dehydro-TxB2 plus 11-dehydro-2,3-dinor-TxB2) cannot be used interchangeably with assays that measure 11-dehydro-TxB2 alone, even with utilization of a correction factor.

Furthermore given the significant difference observed in 11-dehydro-TxB2 plus 11-dehydro-2,3-dinor-TxB2, but not 11-dehydro-TxB2 alone, between those with vs. those without atherothrombotic MI, the present data suggest that 11-dehydro-2,3-dinor-TxB2 may be integral to the process of atherothrombosis. Validation of this relationship might lead to further understanding of atherothrombosis, which would be of considerable clinical significance. Additionally, a biomarker that could differentiate between patients with and without atherothrombotic MI would be of significant clinical importance, because all the current ACS biomarkers measure myocardial necrosis and not the actual cause and therapeutic target itself: atherothrombosis.

There are several plausible mechanisms to explain TxA2 production, in the absence of platelet COX-1 activity, in cardiac patients. Increased inflammation or oxidative stress may induce COX-2 activity, thereby generating TxA2. Although aspirin irreversibly inhibits platelet COX-1, the serum half-life of aspirin is only approximately 20 min, after which nucleated cells (endothelial, macrophages) can produce new COX-1. In addition, several cell types other than platelets can produce thromboxane synthase, which could allow for direct TxA2 production. Finally, metabolic intermediates that do not require COX-1 processing can be produced in other cells and utilized by platelets to produce thromboxane despite the absence of functional COX-1. Identifying the source of TxA2 production, in the absence of platelet COX-1 activity, continues to be an area of active research. Given the differences reported here among 3 assays used to measure “11-dehydro-TxB2”, however, the type of assay used must be carefully considered when evaluating TxA2 production, in the absence of platelet COX-1 activity. Prior work quantifying TxA2 production, in the absence of platelet COX-1 activity, should be re-evaluated in light of these findings. Furthermore, given that 19.5% of subjects with proven platelet COX-1 inhibition met the criteria for “lack of aspirin effect” according to an FDA-approved monoclonal antibody ELISA measure of “11-dehydro-TxB2”, the utility of this test to assess aspirin response should be further examined.

Study Limitations
Although arachidonic acid-induced platelet aggregation is an accepted standard for evaluating platelet COX-1 activity, it is possible that this technique is not adequately sensitive for detecting reduced, but not completely absent, platelet COX-1 activity. The lack of a pre-mortem gold standard for the diagnosis of coronary atherothrombosis may result in misclassification: such an effect would result in an underestimation of the true biological difference in TxA2 metabolites between those with and those without atherothrombosis.

This limitation was addressed in the present study by using a combination of history, biochemical, and imaging criteria in the study classification scheme. We created 2 control groups: 1 included all patients presenting for coronary angiography who did not meet WHF criteria for acute MI, therefore this group consisted largely of patients with stable CAD and is detailed in Table 2. The second control group included both non-atherothrombotic (type II) MI and stable CAD, limiting the ability to conclude that the difference in these groups was due to atherothrombosis alone.

The limited sample size precluded direct comparison of study atherothrombotic and study non-atherothrombotic MI alone. Furthermore, the small sample size precluded a detailed multivariate analysis of potential confounders and limited the power to demonstrate a true difference in TxB2: (if it truly exists in these cohorts), including factors that are known to affect TxB2: production, such as smoking and statin use. Nevertheless, this study had a >98% power for detecting a 25% difference in 11-dehydro-TxB2 and 11-dehydro-2,3-dinor-TxB2: plus 11-dehydro-TxB2: between the studied groups, with the 3 assays used.

Conclusions
Differences in the measurement of TxA2: production, in the absence of platelet COX-1 activity, between those with vs. without atherothrombotic MI was not observed when TxA2: generation was assessed on 11-dehydro-TxB2: production alone (polyclonal ELISA or LC-MS/MS assays), but differences were observed when TxA2: generation was assessed by measurement of 11-dehydro-TxB2: plus 11-dehydro-2,3-dinor-TxB2: (monoclonal ELISA assay). Therefore, 11-dehydro-2,3-dinor-TxB2: may play an important role in the pathophysiology of atherothrombosis, particularly in individuals on adequate aspirin therapy. Given the great clinical importance of the accurate diagnosis and treatment of atherothrombosis, investigation of such an analyte is warranted. The substantial differences identified in the measurement of TxA2: generation via 3 commercially available assays raises important questions concerning the assessment of COX1-independent TxA2: generation and calls into question the validity of an FDA-approved monoclonal antibody ELISA to assess aspirin responsiveness.

Acknowledgments
We thank all the patients who volunteered to participate in this study. Funding sources: Johns Hopkins Ciccarone Center Research Award; Cole Family Clinical Research Award, Johns Hopkins University. This publication was made possible by Grant no. UL1 RR 025005 from the National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH), and NIH Roadmap for Medical Research. Its contents are solely the responsibility of the authors and do not necessarily represent the official view of NCRR or NIH. Information on NCRR is available at
Disclosures

Dr DeFilippis serves on a study adjudication committee for Radiometer America to determine clinical truth (diagnosis of study participants) and receives grant support for a research project unrelated to this work from the American Heart Association. Dr Thomas Kicler is on the advisory board for Merck and receives royalties from Siemens for the invention of a coagulation test. Dr Jaffe acknowledges that he has or does consults for most of the major diagnostic companies but not those whose assays were used in this study.

References

1. Rosamond W, Flegel K, Friday G, Furie K, Go A, Greenland K, et al. Heart disease and stroke statistics -- 2007 update: A report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* 2007; 115: e69 – e171.

2. Angiolillo DJ, Ueno M, Goto S. Basic principles of platelet biology and clinical implications. *Circ J* 2010; 74: 597 – 607.

3. Davi G, Patrono C. Platelet activation and atherothrombosis. *N Engl J Med* 2007; 357: 2482 – 2494.

4. Patrono C, Cibattoni G, Pugliese F, Pierucci A, Blair IA, FitzGerald GA. Estimated rate of thromboxane secretion into the circulation of normal humans. *J Clin Invest* 1986; 77: 590 – 594.

5. Patrono C, Coller B, FitzGerald GA, Hirsh J, Roth G. Platelet-active drugs: The relationships among dose, effectiveness, and side effects: The Seventh ACCP Conference on Antiplatelet and Thrombolytic Therapy. *Chest* 2004; 126: 2345 – 2645.

6. Patrono C, Garcia Rodriguez L.A, Landolfi R, Ragni C. Low-dose aspirin for the prevention of atherothrombosis. *N Engl J Med* 2005; 353: 2373 – 2383.

7. Patrignani P, Filabozzi P, Patrono C. Selective cumulative inhibition of platelet thromboxane production by low-dose aspirin in healthy subjects. *J Clin Invest* 1982; 69: 1366 – 1372.

8. Pamukcu B. A review of aspirin resistance: definition, possible mechanisms, detection with platelet function tests, and its clinical outcomes. *J Thromb Thrombolysis* 2007; 23: 213 – 222.

9. Gluckman TJ, McLean RC, Schulman SP, Kieckers TS, Shapiro EP, Conte JV, et al. Effects of aspirin responsiveness and platelet reactivity on early vein graft thrombosis after coronary artery bypass graft surgery. *J Am Coll Cardiol* 2011; 57: 1069 – 1077.

10. Gurbel PA, Blden KP, DiChiera J, Newcomer J, Weng W, Neerchal KI. Aspirin resistance: Detection and relevance. *Circulation* 2007; 115: 3156 – 3164.

11. Krasopoulos G, Brister SJ, Beattie WS, Buchanan MR. Aspirin “resistance” and risk of cardiovascular morbidity: Systematic review and meta-analysis. *BMJ* 2008; 336: 195 – 198.

12. Patrono C, Aspirin resistance: Definition, mechanisms and clinical readouts. *J Thromb Haemost* 2003; 1: 1710 – 1713.

13. Eikelboom JW, Hankey GJ, Thom J, Bhatt DL, Steg PG, Montalescot G, et al. Incomplete inhibition of thromboxane biosynthesis by acetylsalicylic acid: Determinants and effect on cardiovascular risk. *Circulation* 2007; 118: 1705 – 1712.

14. Eikelboom JW, Hirsh J, Weitz JJ, Johnston M, Yi Q, Yusuf S. Aspirin-resistant thromboxane biosynthesis and the risk of myocardial infarction, stroke, or cardiovascular death in patients at high risk for cardiovascular events. *Circulation* 2002; 105: 1650 – 1655.

15. Kuliczkowski W, Witkowski A, Polonski L, Watala C, Filipiak K, Budaj A, et al. Interindividual variability in the response to oral anti-platelet drugs: A position paper of the Working Group on antiplatelet drugs resistance appointed by the Section of Cardiovascular Interventions of the Polish Cardiac Society, endorsed by the Working Group on Thrombosis of the European Society of Cardiology. *Eur Heart J* 2009; 30: 426 – 435.

16. Olson MT, Kicler TS, Lawson JA, McLean RC, Jani J, Fitzgerald GA, et al. Effect of assay specificity on the association of urine 11-dehydro thromboxane B2 determination with cardiovascular risk. *J Thromb Haemost* 2012; 10: 2462 – 2469.

17. Aversano T, Aversano LT, Passamani E, Knatterud GL, Terrin ML, Williams DO, et al. Thrombolysis therapy vs primary percutaneous coronary intervention for myocardial infarction in patients presenting to hospitals without on-site cardiac surgery: A randomized controlled trial. *JAMA* 2002; 287: 1943 – 1951.

18. Stone GW, Bertrand M, Colombo A, Dangas G, Farkouh ME, Feit F, et al. Acute Catheterization and Urgent Intervention Triage strategY (ACUTY) trial: Study design and rationale. *Am Heart J* 2004; 148: 764 – 775.

19. US Department of Health and Human Services FaDA. AspirinWorks Test Kit [11 dehydro thromboxane B2] (urine). http://www.accessdatafdagov/scripts/cdrh/cfdocs/cfCLIA/Detailcfm?ID=10914. (accessed October 1, 2010).

20. Cayman Chemical Company. Aspirin effect - Detection Kit (11-dehydro Thromboxane B2 - monoclonal). Catalog no. 10010153. Package inserts 1: 1 – 11.

21. Cayman Chemical Company. 11-dehydro-TXB2: ELA kit (polyclonal). Catalog no.: 519501. Package insert 519501: 2009; 1 – 32.

22. Armstrong PC, Truss NJ, Ali FY, Dhanji AA, Vojnovic I, Zain ZN, et al. Aspirin and the in vitro linear relationship between thromboxane A2-mediated platelet aggregation and platelet production of thromboxane A2. *J Thromb Haemost* 2006; 8: 1933 – 1943.

23. Christie D. Platelet function testing by aggregometry: Approved guidelines. *Clin Lab Standards Inst* 2008; HS 8-A: 28 (31): 17 – 19.

24. Zack PM, Ischinger T, Aker UT, Dincer B, Kennedy HL. The occurrence of angiographically detected intracoronary thrombus in patients with unstable angina pectoris. *Am Heart J* 1984; 108: 1408 – 1412.

25. Thygesen K, Alpert JS, White HD, Jaffe AS, Apple FS, Galvani M, et al. Universal definition of myocardial infarction. *Circulation* 2007; 116: 2634 – 2653.

26. Wallentin L. P2Y(12) inhibitors: Differences in properties and mechanisms of action and potential consequences for clinical use. *Eur Heart J* 2009; 30: 1961 – 1977.

27. Cipollone F, Cibattoni G, Patrignani P, Pasquali M, Di Gregorio D, Bucciarelli T, et al. Oxidant stress and aspirin-insensitive thromboxane biosynthesis in severe unstable angina. *Circulation* 2000; 102: 1007 – 1013.

28. Fitzgerald DJ, Roy L, Catella F, FitzGerald GA. Platelet activation in unstable coronary disease. *N Engl J Med* 1986; 315: 983 – 989.

29. Vejar M, Fragasso G, Hackett D, Lipkin DP, Maseri A, Born GV, et al. Dissociation of platelet activation and spontaneous myocardial ischaemia in unstable angina. *Thromb Haemost* 1990; 63: 163 – 168.

30. Catella F, FitzGerald GA. Paired analysis of urinary thromboxane B2 metabolites in humans. *Thromb Res* 1987; 47: 647 – 656.

31. Catella F, Healy D, Lawson JA, FitzGerald GA. 11-Dehydrothromboxane B2: A quantitative index of thromboxane A2 formation in the human circulation. *Proc Natl Acad Sci USA* 1986; 83: 5861 – 5865.

32. Cibattoni G, Pugliese F, Pierucci A, Simonetti BM, Patrono C. Fractional conversion of thromboxane B2 to urinary 11-dehydrothromboxane B2 in man. *Biochim Biophys Acta* 1989; 992: 66 – 70.

33. Patrignani P. Aspirin insensitive eicosanoid biosynthesis in cardiovascular disease. *Thromb Res* 2003; 110: 281 – 286.

34. Ziegler BK, Kristensen SD, Vissinger H, Jensen HK, Nielsen HK, Husted SE. Incomplete thromboxane inhibition with 100mg of intravenous acetylsalicylic acid in patients with acute ST elevation myocardial infarction: A placebo-controlled pilot trial. *Thromb Res* 2001; 104: 175 – 180.

35. Patrignani P, Rocca B, Aspirin: Promise and resistance in the new millennium. *Arterioscler Thromb Vasc Biol* 2008; 28: s25 – s32.

36. Nusing R, Ulrich V. Immunoquantification of thromboxane synthesis in human tissues. *Eicosanoids* 1990; 3: 175 – 180.

37. Karim S, Habib A, Levy-Toledano S, Maclouf J. Cyclooxygenase-1 and -2 of endothelial cells utilize exogenous or endogenous arachidonic acid for transcellular production of thromboxane. *J Biol Chem* 1996; 271: 12042 – 12048.

Supplementary Files

Supplementary File 1

Supplementary Files are available with the full text of this article, available online at http://dx.doi.org/10.1253/circ.12-1421

Table S1

Table S1. Study inclusion and exclusion criteria

Please find supplemental file(s): http://dx.doi.org/10.1253/circ.12-1421