Risk Factors for Nonplatelet Thromboxane Generation After Coronary Artery Bypass Graft Surgery

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Kakouros, Nikolaos; Nazarian, Saman; Stadler, Patrizia B.; Kickler, Thomas; and Rade, Jeffrey J., "Risk Factors for Nonplatelet Thromboxane Generation After Coronary Artery Bypass Graft Surgery" (2016). Open Access Articles. 2884.
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Keywords
aspirin, isoprostane, oxidative stress, thrombosis, thromboxane

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Risk Factors for Nonplatelet Thromboxane Generation After Coronary Artery Bypass Graft Surgery

Nikolaos Kakouros, MBBS, PhD; Susanna M. Nazarian, MD, PhD; Patrizia B. Stadler, PhD; Thomas S. Kickler, MD; Jeffrey J. Rade, MD

Background—Persistent thromboxane (TX) generation while receiving aspirin therapy is associated with an increased risk of cardiovascular events. The Reduction in Graft Occlusion Rates (RIGOR) study found that aspirin-insensitive TXA2 generation, indicated by elevated urine 11-dehydro-TXB2 (UTXB2) 6 months after coronary artery bypass graft surgery, was a potent risk factor for vein graft thrombosis and originated predominantly from nonplatelet sources. Our goal was to identify risk factors for nonplatelet TXA2 generation.

Methods and Results—Multivariable modeling was performed by using clinical and laboratory variables obtained from 260 RIGOR subjects with verified aspirin-mediated inhibition of platelet TXA2 generation. The strongest variable associated with UTXB2 6 months after surgery, accounting for 47.2% of the modeled effect, was urine 8-iso-prostaglandin (PG)F2α, an arachidonic acid metabolite generated nonenzymatically by oxidative stress (standardized coefficient 0.442, P<0.001). Age, sex, race, lipid therapy, creatinine, left ventricular ejection fraction, and aspirin dose were also significantly associated with UTXB2 (P<0.03), although they accounted for only 4.8% to 10.2% of the modeled effect. Urine 8-iso-PGF2α correlated with risk of vein graft occlusion (odds ratio 1.67, P=0.001) but was not independent of UTXB2. In vitro studies revealed that endothelial cells generate TXA2 in response to oxidative stress and direct exposure to 8-iso-PGF2α.

Conclusions—Oxidative stress–induced formation of 8-iso-PGF2α is strongly associated with nonplatelet thromboxane formation and early vein graft thrombosis after coronary artery bypass graft surgery. The endothelium is potentially an important source of oxidative stress–induced thromboxane generation. These findings suggest therapies that reduce oxidative stress could be useful in reducing cardiovascular risks associated with aspirin-insensitive thromboxane generation. (J Am Heart Assoc. 2016;5:e002615 doi: 10.1161/JAHA.115.002615)

Key Words: aspirin • isoprostane • oxidative stress • thrombosis • thromboxane
6 months after surgery, respectively. Further, UTXB$_2$ $\geq$450 pg/mg creatinine measured 6 months after CABG was associated with a 2.6-fold increased risk of vein graft thrombosis compared with levels of $<450$ pg/mg creatinine.

These data indicate that a substantial percentage of patients taking aspirin continue to generate TXA$_2$ 6 months after CABG that originates from predominantly nonplatelet pathways and is associated with an increased risk of early vein graft thrombosis. The source and stimuli for nonplatelet TXA$_2$ generation in patients with cardiovascular disease are largely unknown. The goal of this study was to use multivariable modeling to identify factors associated with nonplatelet TXA$_2$ generation.

Materials and Methods

Subjects

The Reduction in Graft Occlusion Rates (RIGOR) study was a multicenter observational study of 368 subjects undergoing first-time CABG between 2003 and 2006 that was designed to investigate the association between thrombotic risk factors and early saphenous vein graft occlusion. Patients were enrolled between October 2003 and October 2006 at 4 participating institutions: Johns Hopkins Hospital, Baltimore, MD; Christiana Hospital, Christiana, DE; Peninsula Regional Medical Center, Salisbury, MD; and Walter Reed Army Hospital, Washington, DC. Institutional human subject research review board approval was obtained at all participating sites, and all subjects provided written consent. A detailed description of the study design, patient characteristics, and principal findings has been previously published.$^{5-7}$ Patients $\geq$18 years of age undergoing first-time CABG with implantation of at least 1 saphenous vein graft were eligible for enrollment. Those with an anticipated requirement for postoperative oral anticoagulation or antiplatelet therapy other than aspirin were excluded, although those prescribed these agents for unforeseen postoperative conditions (eg, atrial fibrillation) continued in the study. All patients were administered aspirin (300–325 mg) within 24 hours of surgery. At hospital discharge, patients were given a supply of 325 mg enteric-coated aspirin and instructed to take 1 tablet daily for 6 months unless directed otherwise by their physician. Pill counts were performed at each postoperative encounter. Demographic, historical, procedural, clinical, and laboratory data were recorded for all patients.

Platelet Studies

Platelet-rich plasma was prepared from blood collected in 3.2% citrate by centrifugation at 100 rpm for 10 minutes, and the platelet count was adjusted to 180 000/mm$^3$ by the addition of platelet-poor plasma. Undiluted samples with a platelet count of $<100$ 000/mm$^3$ were excluded from analysis. Impedance platelet aggregometry was performed by stimulation with arachidonic acid (0.5 mmol/L), ADP (20 $\mu$mol/L), epinephrine (50 $\mu$mol/L), and collagen (1 $\mu$g/mL) with use of a Chrono-Log Model 560CA aggregometer. The maximum aggregation response within 5 minutes was recorded in ohms. Subjects were considered to have aspirin-induced suppression of significant platelet COX-1 activity and TXA$_2$ generation if arachidonic acid–induced platelet aggregation was absent as indicated by a value of $\leq$1 $\Omega$ (normal range in our laboratory for aspirin-naïve subjects: 5–17 $\Omega$) based on prior data demonstrating that suppression of platelet TXA$_2$ generation by $\geq$99% is required to suppress arachidonic acid–induced aggregation by 95%.$^8$ Shear-dependent platelet aggregation was measured by using the Platelet Function Analyzer-100$^{10}$ (PFA-100) device (Siemens Healthcare Diagnostics) as previously described$^9$ in whole blood collected in 3.8% citrate. Samples were tested with the collagen/ADP agonist cartridge, which assesses global platelet reactivity but is not affected by aspirin. Samples from subjects with a platelet count $<50$ 000/m$^3$ were excluded from analysis. Samples with nonclosure were assigned a closure time (CT) value of 300 seconds, the maximum measurable by the device.

Measurement of Urine Prostanoids

11-Dehydro-thromboxane B$_2$ (TXB$_2$) was measured in urine (UTXB$_2$) with ELISA and expressed as a ratio to urine creatinine as previously described.$^9$ Aspirin responsiveness based on this assay was defined as UTXB$_2$ $<$400 pg/mg creatinine according to established criteria.$^{10}$

Assessment of Saphenous Vein Graft Patency

Vein graft patency was assessed 6 months after CABG by the use of multidetector computed tomography coronary-angiography as previously described.$^5$ Data from clinically driven invasive coronary angiograms could be used for the primary end point analysis if performed within 6 weeks of the anticipated 6-month follow-up visit or if it was the only assessment of vein graft patency before an adverse clinical end point. Multisegmented grafts were statistically considered as separate vein grafts according to the Society of Thoracic Surgeons criteria. Reconstructed images were analyzed by 2 blinded reviewers and classified as patent (containing stenoses of 0–75%), significantly diseased (containing stenoses of 76–99%), or occluded (containing a 100% stenosis). There was 98% concordance in assessment of vein graft patency between reviewers. In cases of discordance, a third reviewer adjudicated all vein grafts in that patient.
In Vitro Prostanoid Generation

Human umbilical vein endothelial cells were maintained in EGM-2 medium (Lonza) at 37°C under 5% CO₂. Confluent cells in 10-cm plates were stimulated with hydrogen peroxide (Sigma-Aldrich) and 8-isoprostaglandin (PG)F₂α (Cayman Chemical) for 1 hour at the indicated concentrations. Conditioned media were spiked with tetradecuterated 11-dehydro-TXB₂, TXB₂, and 8-iso-PGF₂α (Cayman Chemical) as internal standards before solid phase extraction by using 50-mg BondElut C18 reverse phase cartridges (Agilent Technologies) preconditioned with ethanol and water to concentrate eicosanoid species. Acidified samples (2% formic acid) were loaded and washed sequentially with water, 15% ethanol, and hexane and then eluted with ethyl acetate, dried, and resuspended in 15% acetonitrile. Calibrants were prepared in the same way over a 0.5- to 500-ng/mL concentration range. Liquid chromatography/mass spectrometry (MS)–MS was performed by using a Dionex UltiMate 3000 UHPLC system in line with a TSQ Quantiva triple quadrupole mass spectrometer (Thermo Fisher Scientific). Chromatographic separation was performed with a Kinetex C18 (1.7 μm, 100 Å) 50 × 100-mm column maintained at 40°C. A multistep gradient with (A) water with 0.005% (v/v) acetic acid, pH 5.7, and (B) 5% methanol/95% acetonitrile with 0.005% acetic acid, at a flow rate of 0.6 mL/min was used. After a 15-μL injection, the gradient started at 15% B (0–0.6 minutes), increased to 40% B (0.6–2 minutes), increased to 95% B (2–4 minutes), was maintained at 95% B (4–4.5 minutes), decreased to 15% B (4.5–4.7 minutes), and was maintained at 15% B (4.7–8 minutes). Tandem MS was performed in negative ion mode with spray voltage set at 3.3 kV, ion transfer tube temperature at 356°C, and vaporizer temperature at 420°C. The sheath, auxiliary, and sweep gases were set at 52, 16, and 2 AU, respectively. The following m/z transitions were monitored for quantification: m/z 353.2 → 193.1 (CE 26 eV) and 353.2 → 309.1 (CE 20 eV) for 8-iso-PGF₂α, 357.2 → 197.1 (CE 26 eV) and 357.2 → 313.1 (CE 20 eV) for d₄-iso-PGF₂α, m/z 367.2 → 243.1 (CE 20 eV) and 367.2 → 305.1 (CE 16 eV) for 11-dehydro-TXB₂; m/z 371.2 → 247.1 (CE 20 eV) and 371.2 → 309.1 (CE 16 eV) for d₄-11-dehydro-TXB₂; 369.2 → 169.1 (CE 18 eV) and 369.2 → 195.0 (CE 15 eV) for TXB₂; and 373.2 → 173.1 (CE 18 eV) and 373.2 → 199.0 (CE 15 eV) for d₄-TXB₂. Area ratios of analyte to internal standard were calculated, and concentrations of the samples were determined from the standard curve. All data were processed and integrated in Xcalibur, version 3.0 (Thermo).

Statistical Analysis

UTXB₂ values were normalized by using the natural logarithmic transform. Univariate analyses were performed by using those variables deemed biologically plausible or supported by the literature. Colinearity of covariates was tested by using Fisher exact and Pearson correlation for categorical and continuous variables, eliminating highly collinear covariates based on clinical significance. All predictors with P≤0.15 on univariate analysis were included in a multivariable model that was optimized by using the corrected Akaike Information Criterion. To facilitate comparison of the contribution of independent variables in the multivariable model, the coefficient estimates are reported for independent variables standardized to a variance of 1 (β coefficients). The relative importance of the independent variables was further assessed by dominance analysis. The regression was performed for all possible combinations of the identified predictors, the incremental contribution of each variable to the resulting models was averaged to obtain general dominance (additive decomposition), and conditional dominance evaluations were performed. The independent variables were ranked for their contribution to the multivariable model based on their dominance weights. For vein graft analysis, grafts classified as severely diseased were considered as patent. Univariate analyses were performed on a per-graft basis for the odds of occlusion versus patency for UTXB₂ and urinary (U)8-iso-PGF₂α. Proportions were compared by using a χ² or Fisher’s exact test, and comparisons among groups were made with ANOVA, McNemar, or Kruskal–Wallis testing, as appropriate. Analyses were performed by using Stata/MP 10.0 for Windows (StatCorp). Differences were considered significant when P<0.05.

Results

Study Population Characteristics

Of the 368 subjects undergoing first-time CABG enrolled in the RIGOR study, 299 had measurement of UTXB₂ and platelet reactivity at the time of assessment of vein graft patency 6 months after surgery. Thirty-nine subjects were excluded from the primary analyses: 2 because they had discontinued aspirin, 32 because they were taking additional nonaspirin antiplatelet agents, and 5 because of arachidonic acid–induced platelet aggregation ≥1 Ω despite aspirin therapy. Therefore, 260 subjects taking aspirin monotherapy with verified suppression of platelet COX-1 activity and TXA₂ generation by >99%⁸ were used for the primary analyses. UTXB₂ in this study cohort was non–normally distributed (Figure 1) with a median of 328 pg/mg creatinine (IQR 232–451 pg/mg creatinine). Despite confirmed aspirin-induced suppression of arachidonic acid–induced platelet activation, 82 (31.5%) subjects had UTXB₂ ≥400 pg/mg creatinine, the accepted threshold with this assay for defining putative aspirin nonresponsiveness. Table 1 shows the clinical characteristics of the study cohort as a whole, in subjects.
stratified by UTXB2, and in subjects excluded from the primary analyses.

**Relationship Between Clinical and Laboratory Variables and UTXB2**

To identify potential stimuli and sources for nonplatelet thromboxane generation, univariate analyses were used to explore associations of a wide array of demographic, clinical, and laboratory variables to UTXB2 expressed as a continuous variable (Tables 2 and 3). A multivariable model (fit statistic of 0.44, $P<0.0001$) was then constructed to identify independent predictors of UTXB2 (Table 4). The strongest predictor of UTXB2, accounting for nearly half of the modeled effect, was U8-iso-PGF$_{2 \alpha}$, an isoprostane formed by nonenzymatic metabolism of arachidonic acid under conditions of oxidative stress. $^{12}$ Figure 2 shows the high degree of correlation between normalized values of U8-iso-PGF$_{2 \alpha}$ and UTXB2 in the study population. Age, race, and sex were also independently associated with UTXB2 and accounted for $\approx 10\%$ of the modeled effect, while lipid therapy (predominantly statins), renal function, left ventricular function, and aspirin dose contributed to lesser degrees.

**Relationship of U8-Iso-PGF$_{2 \alpha}$ to Early Vein Graft Failure**

We previously found that aspirin-insensitive thromboxane generation, defined by elevated UTXB2, was a novel and independent risk factor for vein graft thrombosis 6 months after CABG. $^{5}$ Given that U8-iso-PGF$_{2 \alpha}$ is a major determinant of UTXB2, we investigated whether there was a direct relationship of the former to early graft thrombosis. Stratification of RIGOR subjects, regardless of antiplatelet use or aspirin responsiveness, by tertile of U8-iso-PGF$_{2 \alpha}$, revealed a proportional increase in the prevalence of vein graft occlusion (Figure 3), and normalized U8-iso-PGF$_{2 \alpha}$ correlated with graft occlusion when considered on a per-graft basis (odds ratio 1.67, $P=0.001$). U8-iso-PGF$_{2 \alpha}$ was not, however, an independent predictor of vein graft occlusion in multivariable modeling when UTXB2 was included as a variable (data not shown), indicating the primacy of the latter.

**Relationship Between Endothelial Thromboxane Generation and Oxidative Stress**

The strong association between U8-iso-PGF$_{2 \alpha}$ and UTXB2 highly suggests, but does not in itself prove, a causal relationship between oxidative stress and nonplatelet TXA$_2$ generation. Because the endothelium is a potential major source of nonplatelet TXA$_2$ generation in vivo, we determined the effect of oxidative stress and direct stimulation with 8-iso-PGF$_{2 \alpha}$ on endothelial TXA$_2$ generation. Exposure of human umbilical vein endothelial cells to hydrogen peroxide resulted in a dose-dependent increase in the concentration of 8-iso-PGF$_{2 \alpha}$, TXB2, and 11-dehydro-TXB2 in the conditioned media, indicative of cellular TXA$_2$ generation (Figure 4A). Further, direct stimulation of human umbilical vein endothelial cells with 8-iso-PGF$_{2 \alpha}$ results in endothelial TXA$_2$ production (Figure 4B), establishing a mechanistic link among oxidative stress, 8-iso-PGF$_{2 \alpha}$ formation, and nonplatelet TXA$_2$ generation.

**Discussion**

The major findings of this study are that (1) oxidative stress–induced formation of 8-iso-PGF$_{2 \alpha}$ is the strongest variable associated with nonplatelet TXA$_2$ generation in patients 6 months after CABG; (2) age, sex, race, lipid therapy, aspirin dose, and kidney and left ventricular function are also independently associated with nonplatelet TXA$_2$ generation, though to a much lesser degree; (3) U8-iso-PGF$_{2 \alpha}$ directly correlates with incidence of early vein graft thrombosis, but its predictive power is not independent of UTXB2; and (4) endothelial cells are capable of generating TXA$_2$ in response to both oxidative stress and direct stimulation with 8-iso-PGF$_{2 \alpha}$, thus representing a potential source of nonplatelet TXA$_2$ generation in vivo.

Platelets are the predominant source of TXA$_2$ generation in healthy individuals, and measurement of stable TXA$_2$ metabolites in the urine has been used clinically as an indicator of the antiplatelet effects of aspirin. Substudies from the Heart Outcomes Prevention Evaluation (HOPE) and the Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilization, Management and Avoidance (CHARISMA) trials found that in...
Table 1. Baseline, Operative, and Postoperative Characteristics of the 260 Study Subjects Stratified by UTXB2 and the 39 Excluded Subjects

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>&lt;400 pg/mg Creatinine</th>
<th>≥400 pg/mg Creatinine</th>
<th>P Value</th>
<th>Total Included</th>
<th>Total Excluded</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>178</td>
<td>82</td>
<td>0.07</td>
<td>260</td>
<td>39</td>
<td>0.44</td>
</tr>
<tr>
<td>Age, y</td>
<td>63 (55–69)</td>
<td>66 (57–73)</td>
<td></td>
<td>63 (56–71)</td>
<td>63 (59–72)</td>
<td>0.02</td>
</tr>
<tr>
<td>Male sex</td>
<td>153 (86%)</td>
<td>58 (71%)</td>
<td>0.006</td>
<td>211 (81%)</td>
<td>25 (64%)</td>
<td>1.0</td>
</tr>
<tr>
<td>White race</td>
<td>17 (10%)</td>
<td>18 (22%)</td>
<td>0.01</td>
<td>35 (13%)</td>
<td>5 (13%)</td>
<td>1.0</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>29 (26–33)</td>
<td>28 (26–33)</td>
<td>0.87</td>
<td>29 (26–33)</td>
<td>26 (24–30)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Medical history, n

| Hypertension                        | 148 (83%)              | 65 (79%)              | 0.49    | 213 (82%)     | 32 (84%)      | 0.82    |
| Dyslipidemia                        | 150 (85%)              | 65 (79%)              | 0.29    | 215 (83%)     | 34 (89%)      | 0.48    |
| Diabetes                            | 56 (32%)               | 39 (48%)              | 0.018   | 95 (37%)      | 11 (29%)      | 0.47    |
| Heart failure                       | 16 (9%)                | 18 (22%)              | 0.006   | 34 (13%)      | 4 (10%)       | 0.80    |
| Peripheral/cerebrovascular disease  | 28 (16%)               | 18 (22%)              | 0.226   | 46 (18%)      | 8 (21%)       | 0.66    |
| Atrial fibrillation                 | 5 (3%)                 | 3 (4%)                | 0.71    | 8 (3%)        | 4 (10%)       | 0.06    |
| Current tobacco use                 | 33 (19%)               | 27 (33%)              | 0.017   | 60 (23%)      | 11 (28%)      | 0.55    |
| Myocardial infarction               | 64 (36%)               | 39 (48%)              | 0.08    | 103 (40%)     | 18 (46%)      | 0.49    |
| Prior PCI                           | 40 (22%)               | 12 (15%)              | 0.18    | 52 (20%)      | 9 (23%)       | 0.67    |
| Preoperative LVEF                   | 0.78                   |                       |         |               |               | 0.75    |
| ≤30%                                | 14 (8%)                | 8 (10%)               |         | 22 (8%)       | 3 (8%)        |         |
| 30–50%                              | 59 (33%)               | 29 (35%)              |         | 88 (34%)      | 11 (28%)      |         |
| >50%                                | 105 (59%)              | 45 (55%)              |         | 150 (58%)     | 25 (64%)      |         |
| Urgent/emergent surgery            | 100 (56%)              | 57 (70%)              | 0.06    | 157 (60%)     | 30 (77%)      | 0.052   |
| Euroscore                           | 3 (1–5)                | 4 (3–6)               | 0.004   | 3 (2–5)       | 4 (2–5)       | 0.25    |
| Arterial graft implanted            | 175 (98%)              | 79 (96%)              | 0.38    | 254 (98%)     | 36 (92%)      | 0.10    |
| No. of SVGs per subject            | 0.2                    |                       |         |               |               | 0.03    |
| 1                                   | 48 (27%)               | 20 (24%)              |         | 68 (26%)      | 17 (44%)      |         |
| 2                                   | 70 (39%)               | 41 (50%)              |         | 111 (43%)     | 12 (31%)      |         |
| 3                                   | 44 (25%)               | 12 (15%)              |         | 56 (22%)      | 10 (26%)      |         |
| ≥4                                  | 16 (9%)                | 9 (11%)               |         | 25 (10%)      | 0 (0%)        |         |

Medications at the time of SVG patency assessment

| Aspirin                             | 178 (100%)             | 82 (100%)             | 1.0     | 260 (100%)    | 37 (95%)      | <0.001  |
| Nonaspirin antiplatelet             | 0 (0%)                 | 0 (0%)                | 1.0     | 0 (0%)        | 33 (85%)      | <0.001  |
| Aspirin low dose (<325 mg)          | 11 (6%)                | 10 (12%)              | 0.14    | 21 (8%)       | 12 (31%)      | <0.001  |
| Oral anticoagulation                | 6 (3%)                 | 7 (9%)                | 0.12    | 13 (5%)       | 3 (8%)        | 0.49    |
| β-Blocker                           | 153 (86%)              | 61 (75%)              | 0.035   | 214 (82%)     | 35 (90%)      | 0.36    |
| ACE inhibitor/ARB                   | 113 (63%)              | 47 (57%)              | 0.4     | 160 (62%)     | 26 (67%)      | 0.60    |
| Lipid-lowering agent                | 162 (91%)              | 65 (79%)              | 0.015   | 227 (87%)     | 37 (95%)      | 0.28    |

Values are median (IQR) or n (%). ACE indicates angiotensin-converting enzyme; ARB, angiotensin II receptor blocker; LVEF, left ventricular ejection fraction; PCI, percutaneous coronary intervention; SVG, saphenous vein graft; UTXB2, urinary thromboxane B2.

patients with either established or at high risk for cardiovascular disease who are receiving aspirin therapy, those with UTXB2 in the highest quartile had a 1.66- to 1.80-fold increased risk of death, myocardial infarction, and stroke compared those in the lowest quartile.\(^2,3\) An early interpretation of these results was that aspirin failed to adequately inhibit platelet COX-1 activity in a substantial number of subjects, leading to persistent TXA2 generation, increased
Table 2. Univariate Analyses of the Associations of Subject Demographics, Past Medical History and Medication Use With UTXB2 (Normalized by Natural Log Transformation of pg/mg Creatinine)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Standardized Coefficient*</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female sex</td>
<td>0.225</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age, y</td>
<td>0.203</td>
<td>0.001</td>
</tr>
<tr>
<td>White race (versus nonwhite)</td>
<td>−0.188</td>
<td>0.006</td>
</tr>
<tr>
<td>Obesity (BMI ≥30 kg/m²)</td>
<td>−0.101</td>
<td>0.093</td>
</tr>
<tr>
<td>Medical history</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>−0.031</td>
<td>0.579</td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>−0.141</td>
<td>0.030</td>
</tr>
<tr>
<td>Diabetes</td>
<td>0.110</td>
<td>0.091</td>
</tr>
<tr>
<td>Current tobacco use</td>
<td>0.155</td>
<td>0.011</td>
</tr>
<tr>
<td>Former tobacco use</td>
<td>0.097</td>
<td>0.111</td>
</tr>
<tr>
<td>Myocardial infarction</td>
<td>0.052</td>
<td>0.394</td>
</tr>
<tr>
<td>Percutaneous coronary intervention</td>
<td>−0.054</td>
<td>0.272</td>
</tr>
<tr>
<td>Congestive heart failure</td>
<td>0.140</td>
<td>0.015</td>
</tr>
<tr>
<td>Cerebrovascular disease</td>
<td>0.044</td>
<td>0.524</td>
</tr>
<tr>
<td>Deep venous thrombosis/pulmonary embolus</td>
<td>−0.027</td>
<td>0.436</td>
</tr>
<tr>
<td>Peripheral vascular disease</td>
<td>0.167</td>
<td>0.010</td>
</tr>
<tr>
<td>Chronic obstructive pulmonary disease</td>
<td>0.139</td>
<td>0.016</td>
</tr>
<tr>
<td>Atrial fibrillation</td>
<td>0.038</td>
<td>0.581</td>
</tr>
<tr>
<td>Preoperative LVEF: &lt;30% vs 30-50%</td>
<td>−0.110</td>
<td>0.360</td>
</tr>
<tr>
<td>Preoperative LVEF: &lt;30% vs ≥50%</td>
<td>−0.148</td>
<td>0.211</td>
</tr>
<tr>
<td>Left ventricular ejection fraction (%)</td>
<td>−0.157</td>
<td>0.011</td>
</tr>
<tr>
<td>Euroscore: 0–2 vs 3–5</td>
<td>0.227</td>
<td>0.001</td>
</tr>
<tr>
<td>Euroscore: 0–2 vs ≥6</td>
<td>0.281</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CABG urgency: elective vs urgent or emergent</td>
<td>0.044</td>
<td>0.501</td>
</tr>
</tbody>
</table>

| Medications                                         |                           |         |
| Aspirin dose (81 mg vs higher)                      | −0.187                    | 0.003   |
| Oral anticoagulation                                | 0.124                     | 0.088   |
| β-Blocker                                           | −0.096                    | 0.112   |
| Angiotensin II receptor blocker                     | −0.046                    | 0.488   |
| Angiotensin-converting enzyme inhibitor             | −0.029                    | 0.636   |
| Lipid therapy                                       | −0.195                    | 0.001   |
| Diuretic                                            | 0.046                     | 0.457   |
| Insulin                                             | 0.020                     | 0.726   |
| Insulin sensitizer                                  | −0.004                    | 0.954   |
| Insulin secretagogue                                | 0.064                     | 0.346   |

BMI indicates body mass index; CABG, coronary artery bypass graft surgery; LVEF, left ventricular ejection fraction; UTXB2, urinary thromboxane B2.

*Coefficients are standardized to 1 SD of the predictor. Huber–White sandwich estimates were used to produce robust estimates of variance.

Table 3. Univariate Analyses of the Associations of Laboratory Variables to UTXB2 (Normalized by Natural Log Transformation of pg/mg Creatinine)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Standardized Coefficient*</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematologic parameter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leukocyte count: 4.5–11 × 10⁹ mm⁻³</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>Leukocyte count: ≤4.5 × 10⁹ mm⁻³</td>
<td>0.023</td>
<td>0.702</td>
</tr>
<tr>
<td>Leukocyte count: ≥11 × 10⁹ mm⁻³</td>
<td>0.078</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MCV 80–100 fl</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>MCV &lt;80 fl</td>
<td>0.067</td>
<td>0.354</td>
</tr>
<tr>
<td>MCV &gt;100 fl</td>
<td>0.003</td>
<td>0.925</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>−0.089</td>
<td>0.183</td>
</tr>
<tr>
<td>Red cell distribution width (≤14.5 vs &gt;14.5%)</td>
<td>0.130</td>
<td>0.042</td>
</tr>
<tr>
<td>RDW, %³</td>
<td>0.141</td>
<td>0.024</td>
</tr>
<tr>
<td>Platelet count (&lt;150 vs ≥150 × 10⁹ mm⁻³)</td>
<td>−0.032</td>
<td>0.582</td>
</tr>
<tr>
<td>Reticulocyte (ln %)</td>
<td>−0.083</td>
<td>0.273</td>
</tr>
<tr>
<td>Mean platelet volume (%⁻¹)</td>
<td>−0.050</td>
<td>0.401</td>
</tr>
<tr>
<td>Immature platelet fraction (ln %)</td>
<td>0.055</td>
<td>0.390</td>
</tr>
<tr>
<td>Blood group: 0 vs other</td>
<td>−0.015</td>
<td>0.805</td>
</tr>
<tr>
<td>Rh positivity</td>
<td>0.071</td>
<td>0.341</td>
</tr>
<tr>
<td>Creatinine (−[mg/dL]⁻¹)</td>
<td>−0.166</td>
<td>0.002</td>
</tr>
<tr>
<td>C-reactive protein (&lt;5 vs ≥5 mg/L)</td>
<td>0.139</td>
<td>0.027</td>
</tr>
<tr>
<td>Fibrinogen &lt;390 vs ≥390 mg/dL</td>
<td>0.124</td>
<td>0.049</td>
</tr>
<tr>
<td>vonWillebrand factor (&gt;150% vs ≤150%)</td>
<td>0.192</td>
<td>0.002</td>
</tr>
<tr>
<td>Urine 8-iso-PGF₂α (ln pg/mg creatinine)</td>
<td>0.500</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Urine 8-iso-PGF₂α (&lt;1061 vs ≥1061 pg/mg creatinine)</td>
<td>0.353</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fasting serum insulin (ln μU/mL)</td>
<td>−0.008</td>
<td>0.91</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Impedance platelet aggregation in ohms to</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP (20 μmol/L)</td>
<td>0.041</td>
</tr>
<tr>
<td>Collagen (1 μg/mL)</td>
<td>−0.006</td>
</tr>
<tr>
<td>Epinephrine (50 μmol/L)</td>
<td>0.078</td>
</tr>
<tr>
<td>PFA-100 collagen/ADP (closure time in s)</td>
<td>−0.014</td>
</tr>
<tr>
<td>PFA-100 collagen-epinephrine (closure time in s)</td>
<td>−0.097</td>
</tr>
</tbody>
</table>

MCV indicates  PG, prostaglandin; RDW, ; UTXB2, urinary thromboxane B₂.
*Coefficients are standardized to 1 SD of the predictor. Huber–White sandwich estimates were used to produce robust estimates of variance.

platelet reactivity, and elevated cardiovascular risk. The RIGOR study also found that elevated UTXB2 was associated with increased cardiovascular risk, being a potent and independent risk factor for early vein graft thrombosis.5
Unlike the HOPE and CHARISMA data, which did not measure
platelet-specific TXA₂ generation, the RIGOR data revealed that failure of aspirin to inhibit platelet COX-1 and TXA₂ generation was in fact rare, occurring in <1% of subjects 6 months after surgery. This provided compelling evidence that aspirin-insensitive TXA₂ generation in patients with cardiovascular disease, unlike in healthy individuals, predominantly originates from nonplatelet sources.

The current analysis extends our previous results and identifies oxidative stress as a potentially major stimulus for nonplatelet TXA₂ generation by showing a strong correlation with 8-iso-PGF₂α, an arachidonic acid metabolite formed nonenzymatically in a variety of cell types by free radical oxidation. While frequently used as a marker of oxidative stress, 8-iso-PGF₂α is also a biologically active prostanoid that can bind to and activate cellular thromboxane receptors (see reviews13 and 14). Although it is not as potent a platelet agonist as TXA₂, 8-iso-PGF₂α can potentiate platelet activation in response to collagen and ADP as well as directly stimulate vasoconstriction. In 2 small prior studies involving patients with unstable angina and diabetes who were taking aspirin, a correlation between UTXB₂ and 8-iso-PGF₂α was identified, though in neither was the source of TXA₂ generation specifically evaluated. Our analysis not only establishes a strong association between 8-iso-PGF₂α and aspirin-insensitive TXA₂ generation in a larger study cohort but also conclusively demonstrates that the latter originates predominantly from nonplatelet sources.

Mounting evidence suggests that oxidative stress—induced generation of 8-iso-PGF₂α and nonplatelet TXA₂ is more than just linearly associated but is causally linked. Treatment with the antioxidant vitamin E has been shown to reduce both U8-iso-PGF₂α and UTXB₂ levels in aspirin-naïve smokers. More definitive are the findings that fetal porcine cerebral and retinal microvessels generate TXA₂ when incubated with 8-iso-PGF₂α, an effect that is blocked by indomethacin. Our data not only confirm this finding in macrovascular endothelial cells but also reveal that oxidative stress itself can be a primary stimulus for endothelial TXA₂ generation. Whether this effect is mediated by the endothelial

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Table 4. Independent Risk Factors for UTXB₂* After Adjustment of Other Variables by Multivariable Regression Analysis

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Standardized Coefficient</th>
<th>P Value</th>
<th>Dominance Weight</th>
<th>Dominance Ranking</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine 8-iso-PGF₂α (ln pg/mg creatinine)</td>
<td>0.442</td>
<td>&lt;0.001</td>
<td>0.472</td>
<td>1</td>
</tr>
<tr>
<td>Age, y</td>
<td>0.239</td>
<td>&lt;0.001</td>
<td>0.102</td>
<td>2</td>
</tr>
<tr>
<td>Female sex</td>
<td>0.129</td>
<td>0.015</td>
<td>0.093</td>
<td>3</td>
</tr>
<tr>
<td>White race (versus nonwhite)</td>
<td>-0.172</td>
<td>0.009</td>
<td>0.085</td>
<td>4</td>
</tr>
<tr>
<td>Lipid therapy</td>
<td>-0.161</td>
<td>0.004</td>
<td>0.077</td>
<td>5</td>
</tr>
<tr>
<td>Creatinine (−[mg/dL]⁻¹)</td>
<td>-0.152</td>
<td>0.002</td>
<td>0.072</td>
<td>6</td>
</tr>
<tr>
<td>Aspirin dose (81 mg vs higher)</td>
<td>-0.145</td>
<td>0.004</td>
<td>0.052</td>
<td>7</td>
</tr>
<tr>
<td>Left ventricular ejection fraction (%)</td>
<td>-0.113</td>
<td>0.032</td>
<td>0.048</td>
<td>8</td>
</tr>
</tbody>
</table>

PG indicates prostaglandin; UTXB₂, urinary 11-dehydro thromboxane B₂ (pg/mg creatinine).

*Normalized using natural log transform.
generation of 8-iso-PGF$_{2\alpha}$ and autocrine stimulation of cellular thromboxane receptors or involves additional intracellular pathways is an area of active investigation.

Because 8-iso-PGF$_{2\alpha}$ is a stable prostanoid that freely circulates, its biological effects can be widespread and distinct from sources of origin. Inflammatory cells are capable of directly generating TXA$_2$ and 8-iso-PGF$_{2\alpha}$, and patients undergoing cardiac surgery with cardiopulmonary bypass have a marked inflammatory response with an observable increase in U8-iso-PGF$_{2\alpha}$ in the early postoperative period.$^{20-22}$ In the RIGOR study, UTXB$_2$ was significantly higher 3 days after CABG than at 6 months, though only the latter correlated with risk of vein graft occlusion.$^5$ While we did find significant correlations of UTXB$_2$ with white blood cell count, C-reactive protein, or fibrinogen on univariate analyses, none of these variables was independently associated with UTXB$_2$, suggesting that inflammation was not a major stimulus for nonplatelet TXA$_2$ generation 6 months after CABG. It is conceivable that the vein grafts themselves contribute to TXA$_2$ generation given that surgical preparation and pressure-induced distention are known to increase oxidative stress in vein segments.$^{23,24}$ However, we did not observe any correlation between the number of vein graft segments implanted at the time of surgery and either UTXB$_2$ or U8-iso-PGF$_{2\alpha}$ 6 months later (data not shown). The elevated UTXB$_2$ and U8-iso-PGF$_{2\alpha}$ observed in a substantial percentage of the RIGOR study cohort are, therefore, likely due predominantly to underlying cardiovascular disease or its risk factors, rather than effects of the CABG per se. UTXB$_2$ has previously been shown to be elevated in diabetics compared with nondiabetics.$^{16}$ While this was also true in our analysis, diabetes was not found to be an independent predictor of UTXB$_2$ when 8-iso-PGF$_{2\alpha}$ was considered as a variable, suggesting that they are different manifestations of the same pathologic process.

The mechanism by which nonplatelet TXA$_2$ generation could adversely affect cardiovascular risk is currently unknown. TXA$_2$ necessarily acts locally because of a short half-life ($\approx$30 seconds) due to degradation to biologically inert TXB$_2$. Although aspirin-inhibited platelets cannot generate appreciable amounts of TXA$_2$, they could potentially still aggregate in response to locally generated nonplatelet TXA$_2$ to cause thrombosis. However, several pieces of evidence argue against platelet hyperreactivity being a major mediator of cardiovascular risk by nonplatelet TXA$_2$ generation. First, the addition of clopidogrel did not reduce cardiovascular risk in CHARISMA subjects with elevated UTXB$_2$.$^3$ Second, UTXB$_2$ in the RIGOR study cohort was independent of multiple parameters of platelet reactivity, including shear-dependent platelet aggregation and whole blood aggregation performed in response to multiple different agonists (Table 3). Third, we found no correlation between U8-iso-PGF$_{2\alpha}$ and platelet reactivity, suggesting that circulating 8-iso-PGF$_{2\alpha}$ did not significantly “prime” or potentiate platelet aggregation in response to more physiologic agonists (data not shown). Rather than potentiating platelet reactivity, local TXA$_2$ generation could predispose to thrombus formation by altering endothelial thromboresistance. Consistent with this concept are the recent findings that thromboxane receptor activation stimulates tissue factor expression in both endothelial cells and monocytes.$^{25,26}$

A significant finding of our analysis was that U8-iso-PGF$_{2\alpha}$ correlated directly with the incidence of early vein graft thrombosis. This suggests that therapies aimed at reducing oxidative stress might be a viable strategy to reduce nonplatelet TXA$_2$ generation and improve outcomes after cardiac surgery. Antioxidants have been shown to be efficacious at reducing the incidence of postoperative atrial fibrillation.$^{27,28}$ While they have not been evaluated for...
efficacy at reducing graft failure, this is an eminently testable hypothesis.

Our study has several potential limitations. Although we examined a wide array of clinical and laboratory variables associated with UTXB2 generation in subjects 6 months after CABG, presumably at a time when the effects of surgery have subsided, it is possible that the relative contributions of oxidative stress and other identified factors differ in populations with various forms or severity of cardiovascular disease. Although the RIGOR cohort was extensively phenotyped and we evaluated numerous potential variables, the 8 variables identified in the multivariable modeling account for only slightly less than half of the modeled effect on UTXB2. Thus, additional risk factors for nonplatelet TXA2 generation remain to be identified.

In summary, we identified several risk factors for nonplatelet TXA2 generation in subjects 6 months after CABG. Not only was oxidative stress-induced formation of 8-iso-PGF2α, the strongest identified risk factor, but it also directly correlated with risk of early vein graft thrombosis. In vitro studies revealed that macrovascular endothelial cells are capable of generating TXA2 under conditions of oxidative stress and with direct stimulation with 8-iso-PGF2α, not only establishing a mechanistic link between oxidative stress and nonplatelet TXA2 generation but pointing to dysfunctional endothelial cells as a potentially major source. These findings provide valuable insights into the pathobiology of nonplatelet TXA2 generation and identify potential therapeutic strategies for its suppression.

Sources of Funding
The study was supported by the Johns Hopkins Institute for Clinical and Translational Research (funded by UL1 RR025005 from the National Center for Research Resources, National Institutes of Health), funding from the Flight Attendant Medical Research Foundation (Dr Rade), and National Institutes of Health 1KL2RR025006-01 (Dr Nazarian), with mate-

Disclosures
None.

References

DOI: 10.1161/JAHA.115.002615

Journal of the American Heart Association


Risk Factors for Nonplatelet Thromboxane Generation After Coronary Artery Bypass Graft Surgery
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J Am Heart Assoc. 2016;5:e002615; originally published March 15, 2016;
doi: 10.1161/JAHA.115.002615

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