Acute Renal Injury After Renal Artery Stenting

Submitted by

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Acute Renal Injury With Renal Artery Stenting

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INTRODUCTION

Considerable attention has been given to the subject of renal artery stenting for preservation of renal function in patients with atherosclerotic renal artery stenosis. Some studies suggest that revascularization stabilizes or improves function, whereas other studies do not. At the crudest level, there is considerable experimental data that demonstrates a relationship between renal artery perfusion pressure, renal blood flow, and glomerular function. Thus, one might conclude that revascularization of a stenotic renal artery would lead to improvement in renal function.

However, this simple construct is not supported by clinical experience. It is undoubtedly the case that the potential benefit of revascularization is counter-balanced by the inclusion of patients with co-existing parenchymal disease in whom revascularization may have no effect and, as is the subject of the current study, patients in whom revascularization leads to additional renal injury. In the balance then, the immediate outcome of any procedure can be viewed as the sum of the benefit from improved renal blood flow weighed against injury caused by the procedure.

To date, little attention has been directed at the causes of renal injury during renal artery revascularization. This topic is clinically important since our primary responsibility to patients is to avoid harm, and is growing in importance, as strategies, such as atheroembolic protection, are developed to prevent renal injury. In the current study we sought to 1) determine the frequency of renal injury (defined as a Cr rise of 0.5 mg/dl) 1 month after renal stenting, and 2) identify factors associated with renal injury.
LITERATURE REVIEW

Atherosclerotic Renal Artery Stenosis

The principle categories of renal artery disease that can cause renal ischemia clinically are atherosclerosis, fibromuscular dysplasia, and atheroembolism. Atherosclerosis has become the predominant form of clinically important renal occlusive disease (Working Group on Renovascular Hypertension, 1987). Evidence from autopsy and angiographic studies has shown an increasing prevalence of renal artery stenosis, especially in the elderly (Greco and Breyer, 1996). Atherosclerotic renal artery stenosis (ARAS) causes two main syndromes: renovascular hypertension and ischemic nephropathy. Ischemic nephropathy is defined as renal insufficiency that is due to hemodynamically significant renal artery stenosis. Both bilateral and unilateral significant occlusive disease, defined as >60% stenosis, can result in potentially reversible renal failure, salvageable by revascularization (Dean et al., 1991). Most of our knowledge of the prevalence of renal artery disease derives from either autopsy or angiographic studies of patients with renovascular hypertension or extra-renal vascular disease in whom simultaneous evaluation of the renal arteries was performed. ARAS occurs in 10% to 15% of hypertensive patients over the age of 50 years, and in 50% to 60% of elderly patients with hypertension, coronary and peripheral vascular disease, and renal insufficiency (Safian, 2003).

Atherosclerotic renal artery disease can be progressive, and progression can be associated with loss of renal function. Progression can be both anatomical and functional. A recent study conducted by Caps et al. reported that within 5 years, greater than 33% of renal arteries initially classified as normal, and 75% of arteries with less than 60% stenosis at baseline had progressed to high-grade (>60%) stenosis (Caps et al., 1998). A study conducted by Zierler et al. showed
similar results with 48% of the patient population progressing from < 60% to ≥ 60% stenosis within 3 yr. (Zierler et al., 1999).

**Improvement or Stabilization of Renal Function After Revascularization**

Many studies have demonstrated that treatment of ARAS with renal artery stenting may improve or stabilize renal function (Dorros et al., 1998; Kennedy et al., 2003; Watson et al., 2000). Stabilization of renal function can be considered clinically successful given the progressive nature of ARAS (Conlon et al., 2000). Recent advancements in diagnostic techniques such as doppler ultrasonography have given valuable insight into prospectively identifying patients that will benefit from renal artery revascularization (Radermacher et al., 2001). The rational for intervention of ARAS is supported by several studies, which point to a window of opportunity for improved renal function, blood pressure control, or both (Textor, 2003). A recent study conducted at our institution followed 261 patients with significant RAS (defined as a ≥ 60% stenosis and/or a translesional systolic pressure gradient ≥ 20 mm Hg) treated with endovascular stenting (Kennedy et al., 2003). In 87 patients with a CrCL of 40 mL/min or less, 32 patients (37%) showed a clinically significant improvement at last follow-up (23 ± 9 verses 32 ± 12 mL/min), and 26 (30%) remained stable (Kennedy et al., 2003). Additionally, there is a significant association between increased renal function, decreased mortality, and a decrease in the occurrence of adverse events (Kennedy et al., 2003). Henry et al followed 48 patients with RAS suffering from renal dysfunction whom were treated with renal artery stents. Renal function improved in 29%, and remained unchanged in 67% of the patients (Henry et al., 1999).
Fortunately, the incidence of chronic renal failure requiring renal replacement therapy after renal intervention is generally low, and observed more frequently in patients that present with severe renal dysfunction (Harden et al., 1997). However, the incidence of chronic renal insufficiency after renal stenting is significantly higher which is not surprising since a large proportion of patients (44%) present with renal insufficiency (White et al., 1997). The reported rates for worsening renal function range from 18 to 28% (Dorros et al., 1998; Harden et al., 1997; Iannone et al., 1996). While most of these are seen in patients with pre-existing renal dysfunction, in a small number of cases (4%) worsening function has been observed despite the presence of normal renal function prior to revascularization (White et al., 1997). Counter-balancing these are the patients that experience significant improvements in renal function after revascularization. Harden et al. reported a greater than 20% decrease in serum creatinine in 34% of the patients after stenting (Harden et al., 1997). Muray et al. demonstrated improved renal function in 58% of revascularized patients (Muray et al., 2002). After a four year follow up, Dorros et al. reported a significant decrease in serum creatinine (2.0 ± 1.4 mg/dl vs 1.5 ± 0.7 mg/dl) in 78% of the eligible patient population after revascularization (Dorros et al., 1998).

Several groups have assessed the slope of reciprocal Cr, comparing pre-intervention to post-intervention (Harden et al., 1997; Watson et al., 2000). In each of these observations revascularization appears to retard the apparent decline in GFR. However, this apparent decline in renal function may be episodic. This is not an unreasonable notion since atherosclerotic events are episodic with other atherosclerotic conditions such as myocardial infarction or stroke where long periods are punctuated by discrete adverse events. The change in slope may be more related to the natural history at the time of identification, rather than the effect of the procedure.
**Renal artery revascularization either decreases renal function, or shows no benefit compared to medical therapy**

Limited data suggests that renal artery revascularization may potentially decrease renal function. Procedural mechanisms where renal artery revascularization may induce renal disease progression are through renal infarction or cholesterol embolism (Leertouwer et al., 2000; van de Ven et al., 1999). Randomized trials comparing medical therapy to angioplasty in patients with ARAS have reported no clinically significant difference between the two groups (Plouin et al., 1998; van Jaarsveld et al., 2000). The series by van Jaarsveld found no difference in median serum creatinine and mean creatinine clearance at 12 months (van Jaarsveld et al., 2000). However, after the 3 month follow-up, 22 of the 50 patients randomized to medical therapy were crossed over to angioplasty (van Jaarsveld et al., 2000). Plouin et al. also reported no significant difference in creatinine clearance between the two groups (Plouin et al., 1998). Yet, after the six month follow-up, 21 of the 26 patients randomized to medical therapy crossed over to angioplasty (Plouin et al., 1998). These studies are limited by significant cross-over effects and inadequate patient populations.

**Factors Associated with Renal Injury**

**Contrast Nephropathy**

Angiographic contrast media are known to cause renal injury. However, there is limited data concerning their role in renal injury during percutaneous renal revascularization procedures. A study conducted by Dorros et al. reported that 13% of the patients had episodes of contrast-induced renal failure after renal artery revascularization (Dorros et al., 1998). There are many
risk factors associated with contrast nephropathy. A study conducted by McCullough et al. reported that patients who received less than 100ml of contrast did not require dialysis after exposure (McCullough et al., 1997). Vlietstra et al. showed that patients who suffered from contrast nephropathy had higher pre-procedural creatinine levels (Vlietstra et al., 1996). Additionally, a randomized trial comparing ionic and nonionic contrast media reported that pre-existing renal insufficiency, diabetes, and male gender were associated with an increased risk of nephrotoxicity (Rudnick et al., 1995). The previously mentioned study also showed an increased risk of nephrotoxicity with ionic contrast media (Rudnick et al., 1995). Thus, among the major risk factors for contrast nephropathy are dose of contrast agent, pre-existing renal dysfunction (especially with coexisting diabetes), and use of ionic contrast media. In renal revascularization, the contrast media are directly injected into the renal artery for visualization. As a consequence, the risk of renal injury may be greater. However, thorough data is needed on the effect of contrast agents in ischemic nephropathy.

Several strategies have been advocated to limit the likelihood of contrast-associated renal dysfunction during renal intervention. Most interventionalists would recommend limiting the dose of contrast agents administered. Techniques employed include limiting the number of test injections, using diluted contrast media, and avoidance of additional angiographic procedures concurrently. A study conducted by Tepel et al. showed that acetylcysteine with hydration may prevent contrast nephropathy (Tepel et al., 2000). However, several other studies have failed to demonstrate a benefit. Briguori et al. reported that acetylcysteine might provide protection only when a small volume of contrast is used (Briguori et al., 2002). Furthermore, in a randomized study comparing acetylcysteine, and fenoldopam, no additional benefit was reported when
compared to hydration therapy (Allaqaband et al., 2002). The success of these endeavors on preventing post-procedural contrast nephropathy remains to be established.

**Atheroembolization**

Atheroembolization is a well-described cause of renal dysfunction (Caps et al., 1998; Krishnamurthi et al., 1999; Wright et al., 2002). The best evidence for the role of atheroembolization in the genesis of renal dysfunction comes from two sources: animal experiments and human observations of spontaneous cases. In the former circumstance, atheroembolic material from human specimens have been administered intra-arterially in animal models. A study conducted by Kimura et al. demonstrated that the severity of renal dysfunction depends on the amount of atheroembolic material present (Kimura et al., 1999). In the previously mentioned study, 0.8 mg, 0.4 mg, 0.2 mg, and 0.1 mg of atheroembolic material was injected intra-arterially into the remaining kidney of nephrectomized rats (Kimura et al., 1999). The animals injected with 0.8 mg showed a significant rise in serum creatinine over a twelve week period (Kimura et al., 1999). The remaining groups showed an initial increase in serum creatinine, but no significant change after a two week period (Kimura et al., 1999). Suzuki et al. reported a steady increase in atrophic tubules as well as prominent interstitial fibrosis after intra-arterial-induced microembolism (Suzuki et al., 2001). Thus, these studies demonstrate the occurrence of renal dysfunction, and severity dependent upon the amount of material administered (Kimura et al., 1999; Suzuki et al., 2001). Additionally, the changes associated with patients experiencing spontaneous atheroembolization are well described in autopsy and biopsy specimens from peripheral and renal tissue (Flory, 1945; Krishnamurthi et al., 1999; Scoble and O’Donnell, 1996; Snyder and Shapiro, 1961). Flory reported atheroembolic...
occlusions causing wedge-shaped areas of cortical atrophy in the kidney (Flory, 1945). Snyder and Shapiro correlated atheroembolization to gangrenous changes in lower extremities (Snyder and Shapiro, 1961). Furthermore, the persistence of intravascular cholesterol crystals for long periods of time has been reported in experimental models (Snyder and Shapiro, 1961).

Renal injury associated with atheroembolization is a complex process (Krishnamurthi et al., 1999). Briefly, Cholesterol crystals embedded in the micovasculature of the kidney causes local inflammation and endothelial vascular reaction (Krishnamurthi et al., 1999). This leads to intimal thickening and cellular proliferation resulting in renal dysfunction (Krishnamurthi et al., 1999).

Embolization of material is a well-described complication of revascularization procedures, whether they be surgical or percutaneous (Krishnamurthi et al., 1999; Scoble and O’Donnell, 1996; Scolari et al., 2000; Thadhani et al., 1995). Prior to cardiovascular surgery or angiography, atheroembolic plaque may dislodge from the aorta and embolize to distal arterial beds due to manipulation of the aorta (Thadhani et al., 1995). Atheroemboli can be a consequence of cardiac catheterization, and angioplasty of the coronary bed, renal arteries, or other arterial systems (Scolari et al., 2000). Furthermore, saphenous vein to coronary artery bypass graft angioplasty procedures are limited by a clinically significant occurrence of procedure-related complications, potentially related to distal atheroembolization (Baim et al., 2002). In general, instrumentation of the aorta disrupts atherosclerotic plaque, and exposes the soft cholesterol core of the plaque to the arterial circulation (Scolari et al., 2000). Krishnamurthi et al. showed that atheroembolic renal disease had a significant correlation with decreased long-term survival after revascularization (Krishnamurthi et al., 1999).
**Platelet Activation and Embolization**

There is a considerable amount of data relating platelet activation to complications in coronary revascularization (Chandrasekar and Tanguay, 2000; Kabbani et al., 2001; King, 2000; LeBrenton et al., 1996). However, little attention has been given to the role of platelets in renal revascularization. When a vessel becomes injured, the subendothelium becomes exposed to circulating platelets. Collagen and vonWillebrand factor (vWF) are located within the subendothelial matrix, and initiate the first phase of platelet adhesion (Moroi and Jung, 1998). Platelets bind collagen directly through the interaction of the platelet glycoprotein receptor VI and integrin \( \alpha 2\beta 1 \) (GPIa/IIa) (Moroi and Jung, 1998). The platelet glycoprotein Ib/IX/V complex binds to vWF (Moroi and Jung, 1998). Immediately after the initial adhesion, the platelets change shape, the integrin glycoprotein IIb/IIIa (GPIIb/IIIa) receptors undergo a conformational change, and the alpha and dense granules are released (Shapiro, 1999; Vinogradova et al., 2000). The \( \alpha \)-granules consist of P-selectin, vWF, fibrinogen, fibrinectin, platelet factor 4, and factor-\( \beta \). The dense granules consist of ADP, ATP, serotonin, calcium, and epinephrine. The ligands for the GPIIb/IIIa receptor are fibrinogen and vWF (Payrastre et al., 2000). These ligands create bridges between receptors on adjacent platelets resulting in aggregation (Shattil et al., 1998). Numerous agonists, such as ADP and thromboxane A2, initiate the conformation change in the GPIIb/IIIa receptor (inside-out signaling) allowing it to bind to fibrinogen, and initiate platelet aggregation (Payrastre et al., 2000). Upon binding to fibrinogen, a series of intracellular signaling mediated by the GPIIb/IIIa receptor (outside-in signaling) results in further platelet aggregation (Payrastre et al., 2000).
In the coronary circulation, platelet inhibition with aspirin and glycoprotein IIb/IIIa inhibitors have demonstrated benefit in reducing ischemic events (Wilson and Fergusson, 1999). Aspirin inhibits the production of thromboxane A2, which plays a pivotal role in platelet aggregation (Shapiro, 1999). GpIIb/IIIa inhibitors also prevent platelet aggregation by inhibiting GPIIb/IIIa receptors (King, 2000). Antiplatelet therapy results in reduced rates of abrupt vessel closure and prevention of platelet embolization from the treatment site into the distal microcirculation. However, the role of platelets in renal dysfunction after renal revascularization remains undefined.

There is a strong rationale for platelet-induced injury. Willerson et al. proposed a mechanism for vascular injury associated with thrombus formation in patients with unstable angina (Willerson et al., 1989). Platelet aggregation and vasoconstriction result from the accumulation of platelet mediators at the site of stenosis and endothelial injury as well as a decrease in the local accumulation of vasodilators, (Willerson et al., 1989). This results in platelet aggregation and thrombus formation (Willerson et al., 1989). Stent insertion can cause rupture of atherosclerotic plaque which results in platelet and subendothelial matrix interactions, platelet aggregation, and platelet-leukocyte interactions mediated by surface receptors and adhesive proteins (Becker, 1999). Once activated, platelets degranulate and release vasoactive or prothrombic material, form clumps that subsequently embolize distally, or in extreme cases occlude the treatment site. Each of these cases has the potential to cause renal dysfunction.

Although the efficacy has never been formally tested, treatment with aspirin before and after renal artery interventions is generally accepted as standard of care. Additional anti-platelet
therapy with theinopyridines, such as clopidogel or ticlopidine, have shown promising results in coronary revascularization (CAPRIE Steering Committee, 1996). However, the benefit of glycoprotein inhibitors and theinopyridines in renal revascularization remains to be seen.

Abciximab (ReoPro) is the Fab (fragment antigen binding) fragment of a mouse/human monoclonal antibody (Clutton et al., 2001). Abciximab inhibits platelet function by binding with high affinity to the GPIIb/IIIa receptor thus, preventing the binding of fibrinogen to activated platelets (Clutton et al., 2001). Abciximab is also able to bind to the vitronectin receptor (integrin αvβ3) with similar affinity (Tam et al., 1998). The vitronectin receptor is expressed on platelets, endothelial cells, and smooth muscle cells (Tam et al., 1998). Vitronectin mediates thrombin generation, clot retraction, and smooth muscle cell proliferation (Tam et al., 1998). Thus, Abciximab serves as a potent GPIIb/IIIa inhibitor, and may also prevent vitronectin mediated events.

**Hemodynamic Injury**

Intra-renal hypertension is an important cause of renal injury in several settings including essential hypertension and malignant hypertension. During renal intervention the kidney is exposed to marked increases in intra-glomerular pressure when the stenosis is relieved. Additionally, many of these patients present with persistent and or severe hypertension as an indication, with some patients remaining hypertensive long after the revascularization is completed.
In this setting it is conceivable that the abrupt increase in intra-renal pressure may injure the glomerulus or other intra-parenchymal vessels. With this in mind, treatment with drugs that inhibit the renin angiotensin system, such as angiotensin converting enzyme inhibitors or angiotensin receptor blockers, may be beneficial. With renal ischemia angiotensin II serves to dilate the afferent arteriole and constrict the efferent arteriole in an effort to maintain glomerular perfusion pressure. However, upon the abrupt release of a stenosis this may lead to inappropriately high glomerular pressures and cause injury. Whether this occurs, and if so, can be prevented with RAS inhibition, is an issue that may benefit from future study.

**Restenosis**

Little is known about the effect of restenosis on renal injury after renal artery revascularization. To date, the majority of studies report the occurrence of restenosis, and not its effect on renal function. A study conducted by Lederman et al. reported an angiographic restenosis rate of 21.3% (Lederman et al., 2001). Additionally, they reported that restenosis seemed to be more common in patients with late renal functional deterioration or lack of blood pressure improvement (Lederman et al., 2001). Restenosis may have as much of an effect on ischemic nephropathy as primary stenosis (Beutler et al., 2001).

**Summary**

Renal artery revascularization has the potential for great benefit and terrible harm. The focus of the present study is to analyze all of the aforementioned factors in patients that have undergone renal artery revascularization. We hypothesize that each of these factors alone, or in combination, contribute to renal injury after revascularization. Our hope is that the current study
will provide physicians with a clearer view of the patient population that can benefit from renal artery revascularization, as well as limiting controllable factors that contribute to renal injury.

OVERVIEW

Specific Aim 1: Determine the frequency and causes of renal injury in patients that undergo renal artery revascularization. To evaluate this aim, we will test the following hypotheses: The presence of a AAA, a likely candidate for aortic embolization, is associated with an increased incidence of acute renal injury as measured by an increase in serum Cr of 0.5mg/dl or greater. The occurrence of acute renal injury increases with the amount of contrast media used. The sudden increase in intra-glomerular pressure, once the stenosis is relieved, leads to acute renal injury.

Specific Aim 2: Determine whether renal artery revascularization is associated with platelet activation. Whole blood flow cytometry and 11-dehydro-thromboxane B2 immunoassays will be used to assess platelet activation.

MATERIALS AND METHODS: SPECIFIC AIM 1

In order to provide reliable estimates for the risk of acute renal injury we evaluated two data sets. The first, the ASPIRE II multicenter study, provides a reliable estimate across multiple institutions, but is limited by extensive exclusion criteria that may not be replicated in clinical practice. The second, a single center registry, provides detailed information with less restrictive entry, but is limited by the use of a single site. We evaluated 186 patients enrolled in the ASPIRE II trial, which was an FDA multicenter trial investigating the safety and effectiveness of renal artery stenting after failed angioplasty. We used this data set to evaluate the occurrence
rate of acute renal injury (based on our definition of a 0.5 mg/dl rise in serum Cr at 1 month) as well as to establish the baseline characteristics of factors that may contribute to acute renal injury. The analysis was repeated in a data set of 137 patients from a single institution that were enrolled in a prospective study evaluating the safety and efficacy of renal artery stenting that included patients with abdominal aortic aneurysms.

Methods: ASPIRE II

Patient Selection

After obtaining approval from the institutional review board, patients with atherosclerotic renal artery stenosis that was suboptimally treated with percutaneous transluminal renal artery angioplasty (PTRA) were offered participation in a multicenter trial investigating the safety and effectiveness of renal artery stenting after failed angioplasty. Major exclusion criteria included the following:

- Presence of an abdominal aortic aneurysm > 4.0 cm in diameter
- Total occlusion of the renal artery
- Any known complications following balloon angioplasty
- Advanced renal disease as evidence by serum Cr ≥ 3.0 mg/dl or kidney length < 8 cm

Population

There were 208 patients that underwent renal artery stenting of atherosclerotic renal artery stenosis in a prospective study. Of these 186 patients (89%) had a baseline Cr ≤ 3.0 mg/dl, and 1 month Cr values performed.
Assessment of renal artery stenosis

A significant renal artery stenosis after failed PTRA was defined as a $\geq 50\%$ residual stenosis (visual estimate), $\geq 20$ mmHg peak translesional and/or $\geq 10$ mmHg mean translesional gradient, or Grade D dissection or any dissection with significant compromise in lumen flow. Renal artery stent placement was performed in the standard fashion through the femoral approach using the Palmaz stent.

Data Collected

Follow-up included outpatient clinic visits at 1, 3, 6, 9, and 24 months. At 1, 6, 9, and 24 month visits, BP and Cr were recorded. At the 3 month follow-up, adverse events were recorded. The selection of anti-hypertensive medications was at the discretion of the treating physician.

Methods: Medical College of Ohio Registry

Patient Selection

After obtaining approval from the institutional review board, patients presenting for renal angiography were offered participation in a prospective cohort study evaluating the safety and efficacy of renal artery stenting in the treatment of renal artery stenosis. Patients who gave informed consent were considered eligible for the study.

Population

Between July 1993 and November, 2001, there were 261 patients that underwent renal artery stenting of atherosclerotic renal artery stenosis in a prospective study as previously reported (Kennedy et al., 2003). One-hundred-fifty-three (153) patients who completed their one month
follow-up at our institution comprised the cohort for the current study. Of these 137 patients (90%) had a baseline Cr ≤ 3.0 mg/dl, and had a one month Cr value performed.

*Assessment of Renal Artery Stenosis*

A significant renal artery stenosis was defined as a ≥60% diameter stenosis and/or a translesional systolic pressure gradient of ≥ 20 mm Hg. Stenoses were quantified by using the digital caliper technique (Uehata et al., 1993). Procedural success was defined as a residual stenosis of <30%.

*Renal Artery Stent Procedure*

Renal artery stent placement was performed using standard techniques (Burket et al., 2000). In most cases, stent implantation was performed immediately following angiography. As part of routine clinical practice at our institution, all patients received intravenous hydration prior to the procedure if renal insufficiency was present. Medications administered immediately prior to stent placement included heparin and aspirin. Aspirin, 325 mg per day, was administered indefinitely thereafter. Accessory renal arteries were treated when significant stenoses, as defined above, were present. Clinically indicated repeat renal angiography was performed in 64/261 (25%) patients.

*Data Collected*

As part of routine clinical practice, all patients had a complete cardiovascular history and physical prior to the angiographic procedure as well as orders for assessment of blood pressure
and renal function at one month in order to adjust for medications. The selection of antihypertensive medications was at the discretion of the treating physician.

*Analysis of Blood Pressure and Renal Function*

Blood pressure and renal function were assessed at baseline and each follow-up visit. At least 1 follow-up BP value and medication list were recorded for all patients surviving to the first follow-up visit. Systemic hypertension was defined as BP $\geq 140/90$ (The Sixth Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure, 1997). When multiple BP readings were available during the month before stenting, an average of at least 2 seated BP measurements was used to determine baseline blood pressure.

Creatinine clearance was estimated using the Cockcroft and Gault formula (Cockcroft and Gault, 1976) and normalized to body surface area calculated using the formula, $(\text{CrCl} \times 1.73\text{m}^2)/\text{Body Surface Area}$. When multiple Cr values were available during the month before stenting, an average of at least 2 Cr values was used. Renal insufficiency was defined as CrCl $< 40$ ml/min. Peri-procedural renal injury was defined as an increase in the serum Cr of $\geq 0.5$ mg/dl, compared with the baseline determination as described previously (Hou et al., 1983). The definition of 0.5 mg/dl was selected as $2.5 \times$ the SD of the range in the Beckman autoanalyzer (0.2 mg/dl).

*Adverse Events*

The medical records of patients whose follow-up was completed at our institution were reviewed by a single nurse. Eleven patients (8%) in whom clinical follow-up was not completed at our institution were omitted from this analysis because the initiation of renal replacement therapy
could not be accurately assessed. Additionally, in the 94 patients with normal baseline Cr (defined as Cr < 1.6 mg/dl) five patients (5%) in whom clinical follow-up was not completed at our institution were omitted from this analysis because the initiation of renal replacement therapy could not be accurately assessed. Renal replacement therapy was defined as the use of dialysis or renal transplantation. Deaths were accounted for in the entire cohort through both clinical follow-up and searching the Social Security Death Master Index File, a national registry indexing >95% of U.S. citizen deaths. Adverse renal events are described in Tables III and IV.

**Statistical Analysis**

Univariate analyses were performed with chi-square analysis and logistic regression for categorical data, and $t$-tests for continuous data. The data are presented as mean ± standard deviation. Statistical significance is defined as $p < 0.05$. A multivariate model was created to assess those factors that were associated with acute renal injury, and this model included all factors that were significant at $p < 0.05$ in univariate analysis. Additionally, diabetes and the amount of contrast volume used were forced into the model. Statistical analysis was performed using commercially available software (StatView, version 5.0, Abacus Concepts, Inc.; SAS, version 6.12, SAS Institute, Inc.).

**RESULTS: SPECIFIC AIM 1**

Of the 261 patients in the dataset, there were 137 patients that had a baseline Cr $\leq 3.0$ mg/dl and one month Cr values after renal artery stenting for atherosclerotic renal artery stenosis at our institution. There were 12 excluded for a baseline Cr $> 3.0$ mg/dl, 4 who did not have a follow-up
Cr and 108 who completed one month follow-up at other institutions. Renal injury occurred in 19 of the 137 patients (14%). The characteristics of the 137 patients are described in Table I.

Of the 208 patients enrolled in the ASPIRE II trial, there were 186 patients that had a baseline Cr ≤ 3.0 mg/dl and one month Cr values after renal artery stenting for atherosclerotic renal artery stenosis. Renal injury occurred in 14 of the 200 patients (7%). The characteristics of the 186 patients are described in Table II.

**Predictors of Acute Peri-procedural Injury:**

Of the 137 patients from our institution, by univariate analysis the presence of an un-repaired abdominal aortic aneurysm was more frequent in patients with acute renal injury (6/19 [32%] vs 11/118 [9%], p < 0.01) as was male gender (13/19 [68%] vs 46/118 [39%], p < 0.05). Baseline Cr was also somewhat higher in the patients with acute renal injury (1.6±0.6 vs 1.3±0.5 mg/dl, p < 0.05). Incidence of smoking was somewhat higher in the patients with acute renal injury, although this trend was not significant (12/19 [63%] vs 49/118 [42%], p =0.08). Neither the presence of diabetes (5/19 [26%] vs 36/118 [31%], p = NS) nor contrast volume (166 ± 69 vs 164 ± 92 mg/dl, p = NS) were associated with acute renal injury at one month. Systolic (165 ± 28 vs 171 ± 26 mm Hg, p = NS) and diastolic blood pressure (82 ± 15 vs 81 ± 15 mm Hg, p = NS), as well as the use of ACE inhibitors (10/19 [53%] vs 62/118 [53%], p = NS) were not associated with acute renal injury. By multivariate analysis, the presence of an un-repaired abdominal aortic aneurysm was associated with acute renal injury (OR 5.5 [1.6-19.1], p < 001). No other factor contributed to the model.
Of the 186 patients from the ASPIRE II trial, by univariate analysis baseline Cr was significantly higher in the acute renal injury group (1.7±0.6 vs 1.3±0.5 mg/dl, p < 0.05). Neither male gender (6/14) [43%] vs (63/172) [37%], P = NS), incidence of smoking (3/14) [21%] vs (37/172) [22%], p = NS), nor the presence of diabetes (6/13) [46%] vs (40/172) [23%], P = NS) were shown to be significant factors in the acute renal injury group. By multivariate analysis, baseline Cr (OR 3.3 [1.3-8.3], p < 0.05) was the only factor associated with acute renal injury.

**Adverse Events: Acute Renal Injury**

From the renal stent registry, the need for renal replacement therapy was higher in patients with acute renal injury (2/17 [12%] vs 0/109 [0%], p < 0.001). Death occurred in 11/19 (58%) of patients with acute renal injury, and in 36/118 (31%) without (p < 0.05).

Adverse events are shown in Table III.

In patients with normal baseline serum Cr (defined as a baseline Cr < 1.6 mg/dl), 11/94 (12%) developed acute renal injury. Death occurred in 7/11 (64%) with acute renal injury, and in 18/83 (22%) without (p < 0.05). Renal replacement therapy was higher patients with normal baseline Cr that developed acute renal injury 1/10 (10%) vs 0/79 (0%), p < 0.05).

From the ASPIRE II trial, the acute renal injury group experienced a higher occurrence of renal replacement therapy (4/14 [29%] vs 2/172 [1%], p < 0.001). Death occurred in 3/14 (21%) of patients with acute renal injury, and in 5/172 (3%) without (p < 0.001). Adverse events are shown in Table IV.
Table I. Baseline Characteristics of the Patients Based on Presence of Acute Renal Injury Defined as a Rise in 1 Month Cr of ≥ 0.5 mg/dl From Baseline.

<table>
<thead>
<tr>
<th></th>
<th>Acute Injury (n=19)</th>
<th>No Renal Injury (n=118)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>72±10</td>
<td>70±9</td>
<td>NS</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>13 (68%)</td>
<td>46 (39%)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>White, non Hispanic, n (%)</td>
<td>16 (84%)</td>
<td>109 (92%)</td>
<td>NS</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>5 (26%)</td>
<td>36 (31%)</td>
<td>NS</td>
</tr>
<tr>
<td>Smoking, n (%)</td>
<td>12 (63%)</td>
<td>49 (42%)</td>
<td>NS</td>
</tr>
<tr>
<td>Contrast Volume – mg/dl</td>
<td>166±69</td>
<td>164±92</td>
<td>NS</td>
</tr>
</tbody>
</table>

**Blood Pressure – mm Hg**

<table>
<thead>
<tr>
<th></th>
<th>Acute Injury</th>
<th>No Renal Injury</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic</td>
<td>165±28</td>
<td>171±26</td>
<td>NS</td>
</tr>
<tr>
<td>Diastolic</td>
<td>82±15</td>
<td>81±15</td>
<td>NS</td>
</tr>
<tr>
<td>Pulse pressure</td>
<td>83±18</td>
<td>89±22</td>
<td>NS</td>
</tr>
<tr>
<td>Antihypertensive drugs</td>
<td>2.5±1.1</td>
<td>2.2±1.0</td>
<td>NS</td>
</tr>
<tr>
<td>ACE* inhibitors, n (%)</td>
<td>10 (53%)</td>
<td>62 (53%)</td>
<td>NS</td>
</tr>
<tr>
<td>Beta blockers, n (%)</td>
<td>6 (32%)</td>
<td>59 (50%)</td>
<td>NS</td>
</tr>
<tr>
<td>Diuretics, n (%)</td>
<td>12 (63%)</td>
<td>55 (47%)</td>
<td>NS</td>
</tr>
</tbody>
</table>

**Renal Function**

<table>
<thead>
<tr>
<th></th>
<th>Acute Injury</th>
<th>No Renal Injury</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine clearance – ml/min</td>
<td>49±25</td>
<td>53±25</td>
<td>NS</td>
</tr>
<tr>
<td>Normalized creatinine clearance– ml/min</td>
<td>43±19</td>
<td>50±21</td>
<td>NS</td>
</tr>
<tr>
<td>Serum creatinine – mg/dl</td>
<td>1.6±0.6</td>
<td>1.3±0.5</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Bilateral stenoses, or
<table>
<thead>
<tr>
<th>Condition</th>
<th>Control (n=120)</th>
<th>Cases (n=90)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>solitary kidney stenosis, n (%)</td>
<td>10 (53%)</td>
<td>44 (37%)</td>
<td>NS</td>
</tr>
</tbody>
</table>

**Cardiac Disease**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Control (n=120)</th>
<th>Cases (n=90)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coronary artery disease, n (%)</td>
<td>18 (95%)</td>
<td>95 (81%)</td>
<td>NS</td>
</tr>
<tr>
<td>Congestive heart failure, n (%)</td>
<td>9 (47%)</td>
<td>38 (32%)</td>
<td>NS</td>
</tr>
<tr>
<td>Myocardial infarction, n (%)</td>
<td>9 (47%)</td>
<td>41 (34%)</td>
<td>NS</td>
</tr>
<tr>
<td>AAA, un-repaired n (%)</td>
<td>6 (32%)</td>
<td>11 (9%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>AAA, repaired n (%)</td>
<td>1 (5%)</td>
<td>4 (3%)</td>
<td>NS</td>
</tr>
</tbody>
</table>

**Indication for Renal Angiogram**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Control (n=120)</th>
<th>Cases (n=90)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension, n (%)</td>
<td>17 (89%)</td>
<td>107 (91%)</td>
<td>NS</td>
</tr>
<tr>
<td>Congestive heart failure, n (%)</td>
<td>7 (37%)</td>
<td>27 (23%)</td>
<td>NS</td>
</tr>
<tr>
<td>Renal insufficiency, n (%)</td>
<td>7 (37%)</td>
<td>30 (25%)</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Values are mean±SD or number and percentage of patients. AAA = abdominal aortic aneurysm, ACE = angiotensin converting enzyme
**Table II.** Baseline Characteristics of the Patients Enrolled in the ASPIRE II Trial Based on Presence of Acute Renal Injury Defined as a Rise in 1 Month Cr of $\geq 0.5$ mg/dl From Baseline.

<table>
<thead>
<tr>
<th></th>
<th>Acute Injury (n=14)</th>
<th>No Renal Injury (n=172)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>70±10</td>
<td>70±10</td>
<td>NS</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>6 (43%)</td>
<td>63 (37%)</td>
<td>NS</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>6 (46%)</td>
<td>40 (23%)</td>
<td>NS</td>
</tr>
<tr>
<td>Smoking, n (%)</td>
<td>3 (21%)</td>
<td>37 (22%)</td>
<td>NS</td>
</tr>
</tbody>
</table>

**Blood Pressure – mm Hg**

<table>
<thead>
<tr>
<th></th>
<th>Systolic 159±20</th>
<th>Diastolic 80±13</th>
<th>Number of Antihypertensive drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>168±25</td>
<td>81±13</td>
<td>1 (7%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7 (4%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 (21%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>66 (38%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3 (43%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>50 (29%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 (29%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>49 (29%)</td>
</tr>
</tbody>
</table>

**Renal Function**

<table>
<thead>
<tr>
<th></th>
<th>Serum creatinine – mg/dl 1.7±0.6</th>
<th>Bilateral stenoses 5 (36%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.3±0.5</td>
<td>34 (20%)</td>
</tr>
</tbody>
</table>

* Values are mean±SD or number and percentage of patients.
**Table III.** Adverse Renal Events of Patients Enrolled in the Renal Stent Registry Based on Presence of Renal Injury (n=137).

<table>
<thead>
<tr>
<th></th>
<th>Acute Injury</th>
<th>No Renal Injury</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deceased, n (% of 137)</td>
<td>11 (58%)</td>
<td>36 (31%)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Dialysis, n (% of 126)</td>
<td>2 (12%)</td>
<td>0 (0%)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Table IV. Adverse Renal Events of Patients Enrolled in the ASPIRE II Trial Based on Presence of Renal Injury (n=186).

<table>
<thead>
<tr>
<th></th>
<th>Acute Injury</th>
<th>No Renal Injury</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deceased, n (% of 186)</td>
<td>3 (21%)</td>
<td>5 (3%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Dialysis, n (% of 186)</td>
<td>4 (29%)</td>
<td>2 (1%)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
MATERIALS AND METHODS: SPECIFIC AIM 2

**Platelet Activation Markers**

As previously explained, once platelets are activated, the GPIIb/IIIa receptor changes its conformation, and the alpha and dense granuals are released (Shapiro, 1999; Vinogradova et al., 2000). GPIIb/IIIa serves as a receptor for vWF factor, fibronectin, and viritonectin (Payrastre et al., 2000). P-selectin, a member of the α-granule components, is expressed on the surface of activated platelets (Michelson, 1996). The GPIb-IX-V complex (CD42) binds to vWF on the surface of exposed endothelium in damaged blood vessels. Each of these components of the platelet activation cascade have been proven to be reliable markers of platelet activation (Michelson, 1996; Wenche et al., 1998).

Thromboxane A2, a metabolite of arachidonate, is formed through the platelet internal metabolic pathways, and serves as an effective platelet aggregator (Shapiro, 1999). Thromboxane A2 is ultimately hydrolyzed to 11-Dehydrothromboxane B2 (11-dehydro TXB2) (Catella et al., 1986). 11-dehydro TXB2 is excreted by the kidneys and has been proven to be an appropriate marker of Thromboxane A2 levels, and platelet activation (Catella et al., 1986). Additionally, Catella et al. reported that patients with severe atherosclerosis have an increased level of 11-dehydro TXB2 (Catella et al., 1986).

**Whole Blood Flow Cytometry**

Whole blood flow cytometry (WBFC) has been established as an effective method for assessing platelet activation (Michelson, 1996; Michelson et al., 2000). WBFC allows for the quantitative evaluation of activated platelets. The activation state is determined by the binding of an
activation dependent monoclonal antibody (Michelson et al., 2000). The level of reactivity can also be assessed by using an exogenous agonist such as ADP (Michelson et al., 2000). Before analysis, platelets are labeled with fluorescently-conjugated antibodies. The fluorophores used in our assay are phycoerythrin (PE), fluorescein isothiocyanate (FITC), and peridinin chlorophyll protein (PerCP). The following conjugated mouse antibodies were used to determine platelet activity.

- IgM-FITC and IgG-PE were used as negative controls
- CD42a-PerCP, which is directed against the GPIb-IX-V complex (Michelson et al., 2000).
- PAC1-FITC, which binds to the activated GPIIb/IIIa receptor (Michelson et al., 2000)
- CD62-PE, which is directed against P-selectin (Michelson et al., 2000)

In the flow cytometer, suspended cells pass through a flow chamber (at a rate of 10,000 cells per minute per our protocol), through the focused beam of a laser (Michelson et al., 2000). After fluorescent activation of the fluorophore at the excitation wavelength, a detector processes the emitted fluorescence and light scattering properties of each cell (Michelson et al., 2000).

Our WBFC assay was designed based on the recommendations in Immunophenotypic Analysis of Platelets (Appendix I), and modified by the flow cytometrists at our institution. The following protocol is used:

1. HT buffer, ADP solution, and 1% formalin are brought to room temperature before use.
2. The antibodies for the negative control are titrated as follows:
• The IgM-FITC antibody serves as a negative control for the PAC1-FITC antibody. Therefore, it should be diluted to match the fluorescence of the PAC1 antibody. The PAC1-FITC stock concentration is 25\(\mu\text{g/ml}\). IgM-FITC stock concentration is 50 \(\mu\text{g/ml}\). We found that an IgM-FITC dilution of 1:3 by HT buffer showed the closest consistent match to PAC1.

• The IgG-PE antibody serves as a negative control for the CD62-PE antibody. The IgG-PE stock concentration is 50\(\mu\text{g/ml}\). The CD62-PE stock concentration is 1.5 \(\mu\text{g/ml}\). Therefore, IgG-PE should be diluted 1:32 by HT buffer.

3. The ADP titration is performed in the following manner:

• The ADP stock concentration is 130\(\mu\text{M}\). A 1.3 \(\mu\text{M}\) solution of ADP is prepared by combining 5\(\mu\text{l}\) of ADP stock solution with 495 \(\mu\text{l}\) of HT buffer.

WBFC test samples are analyzed with a Coulter® EPICS® ELITE cytometer, and prepared in the following manner:

• **Negative Control**: 10\(\mu\text{l}\) of IgM-FITC, IgG-PE, CD42a-PerCP, and 10\(\mu\text{l}\) of HT buffer

• **FITC Compensation Control**: 10\(\mu\text{l}\) of PAC1-FITC, 10\(\mu\text{l}\) of 130\(\mu\text{M}\) ADP and 20\(\mu\text{l}\) of HT buffer

• **PE Compensation Control**: 10\(\mu\text{l}\) of CD62-PE, 10\(\mu\text{l}\) of 130\(\mu\text{M}\) ADP, ad 20\(\mu\text{l}\) of HT buffer

• **PerCP Compensation Control**: 10\(\mu\text{l}\) of CD42a-PerCP, 10\(\mu\text{l}\) of 130\(\mu\text{M}\) ADP, and 20\(\mu\text{l}\) of HT buffer
• **Background Activation**: 10μl of PAC1-FITC, CD42a-PerCP, CD62-PE, and 10μl of HT buffer

• **Low Level Activation**: 10μl of PAC1-FITC, CD42a-PerCP, CD62-PE, and 10μl of 1.3μM ADP

• **High Level Activation**: 10μl of PAC1-FITC, CD42a-PerCP, CD62-PE, and 10μl of 130μM ADP

• 25μl of whole blood Diluted 1:10 in HT buffer is added to each test sample

• Samples are mixed by gentle agitation, and incubated for 20 minutes at room temperature.

• **Fixation**: 935 μl of 1% formalin is added to each sample, and the samples are mixed by gentle agitation for 15 minutes. After this final incubation, the samples are ready to be analyzed.

The following steps were taken to ensure quality control:

• Compensation controls are analyzed with each analysis.

• Negative controls are required for each activation sample. The results of the WBFC will be matched against the negative controls in order to limit the number of false positives.

• Low level and high level platelet activation tests are run with 0.2μM and 20μM of ADP (final concentration). This will enable us to determine the minimal and maximal values for platelet activation, receptor expression, and proper reagent function.

The following reagents, antibodies, and blood collection materials were used for WBFC.

The reagents were made according to the *Immunophenotypic Analysis of Platelets* (Appendix I).
• Antibodies IgG-PE, CD42a-PerCP, CD62-PE, and PAC1-FITC were purchased from B.D. Biosciences, CA. and stored at 4°C. IgM-FITC was purchased from Dako Cytomation, CA. and stored at 4°C.

• Sodium Chloride, Potassium Chloride, Magnesium Chloride, Sodium Bicarbonate, Sodium Phosphate, Dextrose, and NALGENE filters were purchased from Fisher Scientific, IL.

• 10% Formaldehyde solution was purchased from Polyscinces Inc. PA.

• HEPES and ADP were purchased from Sigma Diagnostics, MO.

• HEPES buffered saline (HBS) was made according to recommendations from the *Immunophenotypic Analysis of Platelets* (Appendix I), and stored at 4°C for six months.

• Modified HEPES/Tyrodes buffer (HT buffer) was made according to recommendations from the *Immunophenotypic Analysis of Platelets* (Appendix I), and stored at -20°C for one year.

• The 130 µM ADP solution was made according to the *Immunophenotypic Analysis of Platelets* (Appendix I), and stored at -20°C in 1 ml tubes for one year.

• The 1% Formaldehyde solution was prepared according to the *Immunophenotypic Analysis of Platelets* (Appendix I) and stored at 4°C for one month.

• The Vacutainer® Brand Safety-Loc™ blood collection sets were purchased from Becton Dickinson, NJ. The 10cc syringes were also purchased from Becton Dickinson, NJ.

• 12x75 ml culture tubes were purchased from Fisher Scientific, Il.
• PPACK tubes were purchased from Haematologic technologies Inc., VT. And stored at 4°C.

Data
The results are shown as a series of histograms. The levels of GpIIb/IIIa (PAC1) and P-selectin (CD62) expression are compared to the negative control (corresponding antibodies, IgM-FITC and IgG-PE) in order to limit false positive results. Data is recorded as the percentage of platelets (out of a population of 10,000 per our protocol) that stain positive for the PAC1 (GPIIb/IIIa) and CD62 (P-selectin) antibodies. Data is also shown as the mean fluorescence intensity of the antibodies, which corresponds to the amount of platelet surface antigen. When analyzing mean fluorescence, data is shown as the mean fluorescence intensity of the percent positive population and mean fluorescence intensity of the total population of platelets. The data was stored on ZIP® 100 MB disks and entered into a database developed specifically for this study using the File Maker Pro® program.

11-dehydro-TXB2 ELISA
The basic principal underlying this test is the competitive binding of biotinilated and non-biotinylated antigens to the antibodies that are fixed to the wells. The following protocol is used:
• Urine samples were collected over a two hour period at baseline, immediate post-procedure, 12 hours, 24 hours, and 1 month post-procedure. The samples were frozen at -80°C until analysis.
• Sample analysis was performed using the 11-dehydro Thromboxane B2 EIA Kit purchased from Cayman Chemical, MI., and performed according to the manufacturer’s protocol (Appendix II).

**Quality Control**

Quality control was performed according to the manufacturer’s recommendations (Appendix II).

**Data**

Data from the Spectra MAX 250 microplate spectrophotometer was analyzed using the SoftMax Pro version 1.1 software (Molecular Devices, CA). The data was exported into an Excel® spreadsheet and into a FileMaker pro® database designed for this study.

**Blood Collection**

Blood was drawn from the peripheral vein of the arm. The *Immunophenotypic Analysis of Platelets* was used as a guideline for the following protocol (Appendix I):

- A light tourniquet is used.
- A 21-G needle set (Vacutainer® Brand Safety-Lok™ Blood Collection Set and a 10cc syringe are used for the blood draw.
- At baseline and immediately following the procedure; 2cc of blood will be drawn through the blood collection set, a hemostat is placed on the catheter of the blood collection set, and the blood is discarded. Another 10cc syringe is connected to the set, the hemostat is removed, and blood is carefully drawn into the syringe. Blood drawing is stopped when 6cc of blood is collected. The blood collection set is disconnected from the patient, and immediately connected to the PPAC tubes (Haematologic Technologies) for WBFC and aggregometry.
At 12, 24 hours, and one month following the procedure; blood is collected using the blood collection set and a 10cc syringe. The first 2cc of blood is carefully drawn into the syringe, a hemostat is placed on the catheter of the blood collection set, and the syringe with the 2cc of blood is discarded. Another 10cc syringe is connected to the blood collection set, the hemostat is removed, and 3cc of blood is carefully drawn into the syringe. The needle is withdrawn from the patient and immediately inserted into the PPACK tube (Haematologic Technologies) for use in WBFC.

**Selective Renal Vein Sampling**

At baseline and post procedure, a 6F renal double curve catheter was inserted into the right and left renal veins after localization of the selective vein ostial with a non-selective injection of 40 ml of contrast. Cannulation was confirmed with a small injection of 50:50 contrast:saline mixture. Blood was carefully drawn from the right and left renal vein catheters into a 10cc syringe. The blood draw was stopped when 6cc of blood was collected. The blood was immediately transferred into the PPAC tubes (Haematologic Technologies) for WBFC.

**Control Subjects**

After obtaining approval from the institutional review board, blood samples were obtained from 21 healthy subjects in order to determine normal levels of platelet activation (Table VI).
Patient Population

Blood samples for WBFC and urine samples for Thromboxane A2 analysis were collected from patients enrolled in the RESIST study at our institution. The RESIST study is a multicenter trial investigating the safety and efficacy of the AngioGuard® device and Abciximab. The following inclusion criteria is used:

- Age ≥ 18
- 1 or more renal artery stenosis (≥ 50% and < 100% by angiography)
- Lesion length < 11 mm, vessel diameter < 5.5 mm, and distance between aorta and first bifurcation > 30 mm, or
- Lesion length < 15 mm, vessel diameter < 7.5 mm, and distance between aorta and first bifurcation > 35 mm by visual estimate
- Certain exclusion criteria will also apply (Appendix III)

After baseline evaluation, the patient will be randomly assigned to the following four treatment groups:

1. placebo without AngioGuard
2. Abciximab without AngioGuard
3. placebo plus AngioGuard
4. Abciximab plus AngioGuard

At baseline, post procedure, 12 hours, 24 hours, and 1 month following the procedure; WBFC was performed within 20 minuets of blood collection. Urine samples for the 11-dehydro thromboxane B2 ELISA were collected at baseline, immediate post-procedure, and at 12 hours, 24 hours, and 1 month following the procedure. After collection, the samples will be immediately frozen at -80°C until analysis.
**Statistical Analysis**

The primary aim of this component is to determine whether platelets are activated during renal artery intervention. Continuous data analyses were performed with Wilcoxon matched rank tests. The data are presented as mean ± standard deviation. Statistical significance is defined as p < 0.05. Platelet activation is defined as a statistically significant change from the baseline determination. Statistical analysis was performed using commercially available software (StatView, version 5.0, Abacus Concepts, Inc.; SAS, version 6.12, SAS Institute, Inc.).

**Sample size estimate:**

Assuming 0.80 power, and an alpha error of 0.05, we have constructed a table detailing the change in platelet markers that can be detected in our projected sample of 20 subjects (Table V). The following estimates are based on preliminary data obtained from nine patients enrolled in the RESIST study. When analyzing percent positive activation; we estimate that we should be able to detect a minimum difference of 37% for GPIIb/IIIa expression, and 43% for P-selectin expression. For analysis based on the mean fluorescence of the percent positive population of platelets; we estimate that we should be able to detect a minimum difference of 30% for GPIIb/IIIa mean fluorescence, and 16% for P-selectin mean fluorescence. For analysis based on the mean fluorescence of the total platelet population; we estimate that we should be able to detect a minimum difference 10% for GPIIb/IIIa mean fluorescence, and 11% for P-selectin mean fluorescence.
Table V. Detectable Change in Platelet Activation Markers

**Percent Positive Activation**

<table>
<thead>
<tr>
<th>VARIABLE</th>
<th>BASELINE MEAN</th>
<th>SD</th>
<th>EXPECTED DETECTABLE CHANGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPIIb/IIIa</td>
<td>1.83</td>
<td>1.09</td>
<td>2.51 (37%)</td>
</tr>
<tr>
<td>P-selectin</td>
<td>8.64</td>
<td>5.95</td>
<td>3.73 (43%)</td>
</tr>
</tbody>
</table>

**Mean Fluorescence of the Percent Positive Population**

<table>
<thead>
<tr>
<th>VARIABLE</th>
<th>BASELINE MEAN</th>
<th>SD</th>
<th>EXPECTED DETECTABLE CHANGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPIIb/IIIa</td>
<td>3.26</td>
<td>1.58</td>
<td>0.99 (30%)</td>
</tr>
<tr>
<td>P-selectin</td>
<td>2.18</td>
<td>0.56</td>
<td>0.35 (16%)</td>
</tr>
</tbody>
</table>

**Mean Fluorescence of the Total Platelet Population**

<table>
<thead>
<tr>
<th>VARIABLE</th>
<th>BASELINE MEAN</th>
<th>SD</th>
<th>EXPECTED DETECTABLE CHANGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPIIb/IIIa</td>
<td>0.29</td>
<td>0.05</td>
<td>0.32 (10%)</td>
</tr>
<tr>
<td>P-selectin</td>
<td>0.38</td>
<td>0.06</td>
<td>0.34 (11%)</td>
</tr>
</tbody>
</table>
RESULTS: SPECIFIC AIM 2

After analyzing the percent positive population, mean fluorescence of the percent positive population, and mean fluorescence of the total platelet population, we did not detect a significant increase in platelet activation (GPIIb/IIIa baseline vs post, $0.27 \pm 0.06$ vs $0.24 \pm 0.04$, $p = \text{NS}$), (P-selectin baseline vs post, $0.36 \pm 0.09$ vs $0.45 \pm 0.60$, $p = \text{NS}$) (Table VII and Figures 1-2).

We also did not detect a significant increase in platelet activation in patients that were not currently on an anti-platelet regimen (Table VIII). In support of our results, we obtained additional blood samples at baseline and post procedure from both renal veins of three patients that underwent unilateral stenting. The affected renal vein samples did not show a significant increase in platelet activation when compared to samples obtained from the femoral artery or the contra lateral renal vein (Table IX and Figures 3-4). We did detect a significant decrease in the percent positive expression of GPIIb/IIIa in all patients ($2.32 \pm 2.82$ vs $1.16 \pm 1.54$, $p < 0.05$), and in patients not on anti-platelet therapy ($3.00 \pm 0.40$ vs $1.50 \pm 0.90$, $p < 0.05$) (Tables VII-VIII).

There was a significant increase in thromboxane B2 levels at 24 hours post procedure ($13.1 \pm 17.1$ vs $17.6 \pm 17.3$ (pg/ml) per mg/dl Cr, $p < 0.05$) (Table X and Figure 5). The thromboxane data set included 10 patients with renal dysfunction (defined as a serum Cr $\geq 1.6$ at the baseline determination). The patients with renal dysfunction had a significant rise in thromboxane at 24 hours post procedure ($6.0 \pm 5.2$ vs $18.5 \pm 23.6$ (pg/ml) per mg/dl Cr, $p < 0.05$) (Table X and Figure 7). Whereas those without renal dysfunction did not ($16.6 \pm 19.9$ vs $17.2 \pm 13.9$ (pg/ml) per mg/dl Cr, $p = \text{NS}$) (Table X and Figure 6).
Table VI. Assessment of Platelet Activation in Control Subjects

Controls (n = 21)

Percent Positive Activation
GPIIb/IIIa: 1.99 ± 2.52
P-selectin: 11.50 ± 5.26

Mean Fluorescence of the Percent Positive Population
GPIIb/IIIa: 1.88 ± 0.94
P-selectin: 1.90 ± 0.43

Mean Fluorescence of the Total Population
GPIIb/IIIa: 0.24 ± 0.02
P-selectin: 0.34 ± 0.06

Table VII. Assessment of Platelet Activation in Patients

Patients (n = 20)

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Post Procedure</th>
<th>HR12</th>
<th>HR 24</th>
<th>1 Month</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent Positive Activation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPIIb/IIIa</td>
<td>2.32 ± 2.82</td>
<td>1.16 ± 1.54</td>
<td>1.04 ± 1.00</td>
<td>0.95 ± 0.65</td>
<td>1.10 ± 0.90</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>P-selectin</td>
<td>12.20 ± 8.10</td>
<td>9.87 ± 10.00</td>
<td>11.60 ± 11.00</td>
<td>12.40 ± 16.90</td>
<td>13.40 ± 15.00</td>
<td>NS</td>
</tr>
<tr>
<td>Mean Fluorescence of the Percent Positive Population</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPIIb/IIIa</td>
<td>2.19 ± 1.40</td>
<td>2.33 ± 1.28</td>
<td>2.31 ± 0.93</td>
<td>2.35 ± 0.98</td>
<td>2.32 ± 0.65</td>
<td>NS</td>
</tr>
<tr>
<td>P-selectin</td>
<td>1.91 ± 0.63</td>
<td>2.23 ± 2.30</td>
<td>1.63 ± 0.60</td>
<td>2.20 ± 1.07</td>
<td>1.89 ± 0.35</td>
<td>NS</td>
</tr>
<tr>
<td>Mean Fluorescence of the total Population</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPIIb/IIIa</td>
<td>0.27 ± 0.06</td>
<td>0.24 ± 0.04</td>
<td>0.24 ± 0.03</td>
<td>0.23 ± 0.04</td>
<td>0.24 ± 0.04</td>
<td>NS</td>
</tr>
<tr>
<td>P-selectin</td>
<td>0.36 ± 0.09</td>
<td>0.45 ± 0.60</td>
<td>0.39 ± 0.16</td>
<td>0.41 ± 0.27</td>
<td>0.36 ± 0.13</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are mean ± SD
Figure 1.

GPIIb/IIa Mean Fluorescence Intensity of the Total Population  
\( p = \text{NS} \)

Figure 2.

P-selectin Mean Fluorescence Intensity of the Total Population  
\( p = \text{NS} \)
Table VIII. Assessment of Platelet Activation in Patients not on Anti-platelet Therapy

Patients (n = 12)

<table>
<thead>
<tr>
<th>Percent Positive Activation</th>
<th>GPIIb/IIa</th>
<th>Baseline</th>
<th>Post Procedure</th>
<th>HR12</th>
<th>HR 24</th>
<th>1 Month</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P-selectin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GPIIb/IIa</td>
<td>3.00 ± 0.40</td>
<td>1.50 ± 0.90</td>
<td>1.20 ± 0.12</td>
<td>1.10 ± 0.60</td>
<td>0.90 ± 0.60</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>P-selectin</td>
<td>10.80 ± 6.40</td>
<td>6.20 ± 3.90</td>
<td>8.50 ± 6.70</td>
<td>10.40 ± 1.40</td>
<td>11.10 ± 8.00</td>
<td>NS</td>
</tr>
</tbody>
</table>

| Mean Fluorescence of the Percent Positive Population |
|------------------------------------------|-----------|----------|-------------|-------------|-------------|-------------|----------|
| GPIIb/IIa                                 | 2.14 ± 1.21 | 2.15 ± 0.90 | 2.10 ± 1.22 | 1.89 ± 1.00 | 1.73 ± 0.90 | NS |
| P-selectin                                | 2.90 ± 1.30 | 2.23 ± 1.30 | 3.10 ± 2.10 | 2.20 ± 0.80 | 1.89 ± 0.60 | NS |

| Mean Fluorescence of the total Population |
|------------------------------------------|-----------|----------|-------------|-------------|-------------|----------|
| GPIIb/IIa                                 | 0.29 ± 0.07 | 0.25 ± 0.04 | 0.24 ± 0.04 | 0.24 ± 0.03 | 0.25 ± 0.03 | NS |
| P-selectin                                | 0.36 ± 0.07 | 0.38 ± 0.09 | 0.37 ± 0.10 | 0.37 ± 0.08 | 0.38 ± 0.16 | NS |

Values are mean ± SD
Table IX. Femoral Artery, Affected Renal Vein, and Unaffected Renal Vein (n = 3)

<table>
<thead>
<tr>
<th></th>
<th>Femoral Artery</th>
<th>Affected Renal Vein</th>
<th>Unaffected Renal Vein</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Post Procedure</td>
<td>Baseline</td>
<td>Post Procedure</td>
</tr>
<tr>
<td>Percent Positive Activation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPIIb/IIIa</td>
<td>6.93 ± 4.89</td>
<td>3.10 ± 3.70*</td>
<td>6.93 ± 6.19</td>
<td>5.73 ± 4.59</td>
</tr>
<tr>
<td>P-selectin</td>
<td>3.63 ± 4.65</td>
<td>2.70 ± 3.40</td>
<td>11.23 ± 2.31</td>
<td>11.00 ± 8.56</td>
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<td>Mean Fluorescence of the Percent Positive Population</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPIIb/IIIa</td>
<td>1.85 ± 0.41</td>
<td>1.73 ± 0.10</td>
<td>1.94 ± 0.75</td>
<td>1.53 ± 0.21</td>
</tr>
<tr>
<td>P-selectin</td>
<td>2.51 ± 0.37</td>
<td>2.11 ± 0.11</td>
<td>2.11 ± 0.11</td>
<td>1.93 ± 0.25</td>
</tr>
<tr>
<td>Mean Fluorescence of the total Population</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPIIb/IIIa</td>
<td>0.36 ± 0.10</td>
<td>0.30 ± 0.07</td>
<td>0.35 ± 0.09</td>
<td>0.30 ± 0.08</td>
</tr>
<tr>
<td>P-selectin</td>
<td>0.35 ± 0.07</td>
<td>0.30 ± 0.03</td>
<td>0.33 ± 0.03</td>
<td>0.30 ± 0.04</td>
</tr>
</tbody>
</table>

*p < 0.05

Values are mean ± SD
Figure 3.

**GPIIb/IIIa Mean Fluorescence of the Total Population: Affected Femoral Artery, Affected Renal Vein, and Unaffected Renal Vein**

Figure 4.

**P-selectin Mean Fluorescence of the Total Population: Affected Femoral Artery, Affected Renal Vein, and Unaffected Renal Vein**
Table X. 11-dehydro-Thromboxane B2 Levels

11-dehydro-Thromboxane B2 (pg/ml) per mg/dl Cr
Normal Patients and Patients With Renal Dysfunction (n = 30)

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>HR24</th>
<th>1 Month</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Patients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>13.1 ± 17.1</td>
<td>*17.6 ± 17.3</td>
<td>11.2 ± 12.4</td>
</tr>
<tr>
<td>Patients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>With Renal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dysfunction</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

11-dehydro-Thromboxane B2 (pg/ml) per mg/dl Cr
Normal Patients (n = 20)

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>HR24</th>
<th>1 Month</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Patients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>16.6 ± 19.9</td>
<td>17.2 ± 13.9</td>
<td>12.5 ± 13.6</td>
</tr>
</tbody>
</table>

11-dehydro-Thromboxane B2 (pg/ml) per mg/dl Cr
Patients With Renal Dysfunction (n = 10)

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>HR24</th>
<th>1 Month</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>With Renal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dysfunction</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* p < 0.05

Values are mean ± SD
Figure 5.

**11-dehydro-Thromboxane B2: Normal Patients and Patients With Renal Dysfunction**

<table>
<thead>
<tr>
<th>TXB2 (pg/ml) per mg/dl Cr</th>
<th>Mean Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>13.1</td>
</tr>
<tr>
<td>HR24</td>
<td>17.6</td>
</tr>
<tr>
<td>1 Month</td>
<td>11.2</td>
</tr>
</tbody>
</table>

p < 0.05

Figure 6.

**11-dehydro-Thromboxane B2: Normal Patients**

<table>
<thead>
<tr>
<th>TXB2 (pg/ml) per mg/dl Cr</th>
<th>Mean Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>16.6</td>
</tr>
<tr>
<td>HR24</td>
<td>17.2</td>
</tr>
<tr>
<td>1 Month</td>
<td>12.5</td>
</tr>
</tbody>
</table>

p = NS
Figure 7.

11-dehydro-Thromboxane B2: Patients With Renal Dysfunction

Mean Values

TXB2 (pg/ml) per mg/dl Cr

Baseline
HR24
1 Month

p < 0.05

6.0

18.5

8.5
DISCUSSION

As the technique of renal artery stenting has improved, there has been an increased frequency of renal artery revascularization. However, the enthusiasm for revascularization to improve renal function is not shared by many who have highlighted the lack of proven efficacy of revascularization, when compared to medical therapy (van Jaarsveld et al., 2000), and who point out the potential for adverse events with renal artery revascularization (Henry et al., 2001; Iannone et al., 1996). In specific, revascularization-induced renal injury is of considerable concern. Additionally, these episodes may limit the overall benefits of renal artery revascularization on renal function which is likely a balance between improved renal blood flow with revascularization, weighed against renal injury induced by the revascularization procedure.

While it is not yet possible to define the extent of renal injury induced by a revascularization procedure in each patient, it is possible to identify patients with evidence for renal injury early after the revascularization procedure. Interestingly, little data is available on the frequency, causes, and consequence of renal injury associated with stent revascularization. Additionally a definition of acute renal injury is lacking. In the current work we sought to address this issue leveraging previous work to develop a simple definition of a rise of 0.5 mg/dl over a one month interval (Hou et al., 1983). One could consider alternative definitions based upon a percent change in Cr, however, these percentage-based definitions might overestimate the actual occurrence of renal injury at low Cr levels, and under-estimate renal injury at higher baseline levels. To address the latter, we excluded patients with a Cr > 3.0 mg/dl since minor changes in renal function might have resulted in changes in serum Cr that exceed the 0.5 mg/dl threshold.
Acute renal injury occurred in 7% of the population of patients enrolled in the ASPIRE II trial. This is likely a reliable risk estimate, since the data was derived from multiple sites. Importantly, the rate of renal injury was far higher, at 14%, from the patients enrolled at our institution. While a number of factors might explain this, the rate was quite similar, 9%, when the patients with known abdominal aortic aneurysms were excluded.

Importantly, the most potent risk factor for renal injury in the patients from our institution was the presence of an un-repaired abdominal aortic aneurysm. These aneurysms are associated with a low but real risk of major embolization with catheter manipulation which in dramatic cases can lead to bowel ischemia, acute renal failure, limb threatening ischemia, or even death (Hendrickx et al., 1999; Jaeger et al., 1999; Scolari et al., 2000; Theriault et al., 2003). While the current work is not sufficient to make definitive conclusions about the wisdom of stent therapy for renal stenoses in patients with abdominal aneurysms, and it was the minority of patients that experienced renal injury (32%), it suggests caution in this group. Importantly, an increased risk was not observed in patients with repaired aneurysms, suggesting that it is the presence of the aneurysm itself that increases risk.

Recently, the role of atheroembolization as a cause for post-procedural renal dysfunction with renal stenting has grown in importance as atheroembolic protection devices have become available. Two recent reports, one uncontrolled and observational (Henry et al., 2001) and the other with historic controls (Holden et al., 2003), suggest that embolic protection may have some utility in preventing renal injury. Certainly, embolic material has been obtained with the use of these devices. While the current work was unable to assess the role of lesion-related
embolization, a topic that is being evaluated in ongoing studies, it did suggest that embolization from catheter manipulation within the aorta may be an issue of concern.

Since the definition of acute renal injury is based on serum creatinine, it is not surprising that higher baseline Cr levels were associated with increased risk of developing acute renal injury. Clearly, the risk of developing renal injury increases in patients presenting with pre-existing renal dysfunction. This is likely explained by several factors. Firstly, Cr is a non-linear estimator of residual kidney function. Thus, smaller degrees of injury lead to more profound changes when the baseline value is higher. Secondly, dysfunctional kidneys may have less biologic reserve to allow accommodation to additional injury.

Importantly, we did not observe apparent relationships between renal injury and other possible mechanisms for acute renal injury, namely contrast nephropathy and hemodynamic injury. Despite a wide range of contrast dose administered, there was no apparent relationship between the volume of contrast administered and renal injury at one month. While contrast is, or has the potential to be toxic, the use of non-ionic and isosmolar contrast agents may have mitigated the toxic effect (Rudnick et al., 1995). Additionally, it is likely that operators recognized patients at higher risk and thus lowered contrast usage in these high-risk patients, thus masking this relationship. In fact, this relationship was not seen. The amount of contrast volume administered was not related to baseline renal function. Lastly, the current observation does not preclude the possibility that some patients may have experienced a transient rise in Cr related to contrast use that returned to baseline by the one month measure.
Does acute hemodynamic injury play a role in renal injury? It is conceptually possible that the acute rise in intra-parenchymal pressure associated with successful revascularization may lead to injury of the microvasculature, especially in these subjects that are often very hypertensive prior to the procedure. However, we did not observe a relationship between baseline blood pressure and the risk of renal injury, nor did we observe a relationship between the use of angiotensin converting enzyme inhibitors, agents that lower intra-glomerular pressure, and the risk of renal injury. Importantly, other factors that might be expected to effect alterations in renal function over the long-term, namely diabetes and congestive heart failure, did not appear to be associated with a risk of renal injury over the short-term.

By univariate analysis, male gender was found to be a significant predictor of developing acute renal injury in our data set. However, by multivariate analysis male gender was shown to be insignificant. Male gender was also shown to be not significant in the ASPIRE II data set. It seems likely that the association of male gender with developing acute renal injury is a chance finding.

Of importance is the observation that renal injury was associated with increased late adverse event rates, specifically, need for dialysis, and death. While it is not surprising that acute renal injury is associated with late progression to end stage renal disease, it is striking how infrequently renal events were observed when acute renal injury was not present. Specifically, only 2 patients from the ASPIRE II trial and none of the patients from the renal stent registry without renal injury at one month required dialysis during follow-up. If new device or drug technologies are successful at lowering rates of acute renal injury, or we are able to establish
better strategies for patient selection, the rate of late complications of renal intervention may be significantly improved.

Previous work has firmly established the importance of baseline and follow-up renal function on the likelihood of late mortality (Conlon et al., 2001; Dorros et al., 1998; Kennedy et al., 2003). As an extension of those observations, it now appears that acute renal injury is also associated with an increased risk of mortality. While it is beyond the scope of the current work to describe all of the potential mechanisms that relate renal insufficiency to cardiovascular and renal adverse events, it is reasonable to summarize these prior observations, namely that renal insufficiency is associated with activation of adverse neurohumoral pathways (renin-angiotensin-aldosterone systems and sympathetic activation) and acceleration of atherosclerosis through an abundance of mechanisms (Murphy et al., 2002; Rundback et al., 2002). To be sure, the current work does not establish a causal relationship between renal injury and late mortality. Other uncontrolled confounding factors may be responsible. However, the current work does at least suggest that 1) renal injury associated with renal revascularization should be taken seriously, and 2) future studies designed to evaluate strategies to minimize renal injury (such as atheroembolic protection device trials) might represent a unique opportunity to test whether changes in renal function are causally related to mortality.

One could surmise from this analysis that renal injury occurred more frequently in patients with underlying renal insufficiency, and thus are at higher risk for mortality because of the underlying renal dysfunction irrespective of subsequent injury. To address this issue we performed a subset analysis of adverse events in the renal stent registry in patients with normal baseline renal
function (defined as a serum Cr < 1.6 mg/dl). The risk of dialysis and death was increased in patients with acute renal injury. Thus, while baseline renal dysfunction undoubtedly is an important factor for adverse renal events, acute renal injury appears to be independently important.

Despite the abundance of literature linking platelet activation to complications in coronary revascularization, we detected no change in platelet activation in patients undergoing renal revascularization with stenting. There are a number of factors that may explain this unlikely observation. Numerous studies have demonstrated the effectiveness of anti-platelet therapy in coronary revascularization (Chandrasekar and Tanguay, 2000; Kabbani et al., 2001; King, 2000). The minority of patients (40%) were on dual anti-platelet therapy with aspirin and thienopyridines prior to revascularization. It is possible that platelet activation was effectively reduced in patients currently on anti-platelet therapy before renal intervention. However, platelet activation was not observed in patients that were not on an anti-platelet regimen. Additionally, treatment with aspirin both before and after renal artery intervention has become standard of care. Although it is beyond the scope of the current study to formally test the effectiveness of aspirin, it is possible that aspirin effectively inhibited platelet activation.

The renal artery possesses a much larger diameter and higher rate of blood flow compared to the coronary artery. It is conceivable that in the coronary circulation, upon rupture of the endothelium in the coronary artery, circulating platelets are more prone to activation due to greater contact with the vessel wall as a result of lower blood flow, and a smaller volume to
surface area ratio. However, in the renal artery platelets may be less prone to activation due to a larger vessel diameter, and higher rate of blood flow.

Recently, the role of tissue factor in the generation of atherothrombosis has received considerable attention (Badimon et al., 1999; Viles-Gonzalez and Badimon, 2003; Viles-Gonzalez et al., 2004). Tissue factor is located in vascular cells and the lipid core within atherosclerotic plaque (Viles-Gonzalez and Badimon, 2003). Once tissue factor is exposed to the bloodstream, it binds to factor VIIa and initiates the extrinsic pathway of the coagulation cascade (Viles-Gonzalez and Badimon, 2003).

Atherosclerotic plaque within the coronary circulation may contain larger lipid cores in comparison to atherosclerotic plaque within the renal arteries. It has been postulated that renal plaques, which are usually ostial in location, represent an extension of aortic plaque into the renal artery lumen (Reynolds et al., 2004). It is possible that these plaques contain less lipid core. Upon rupture of the proposed larger lipid core in the coronary circulation, this could lead to a greater amount of tissue factor exposed to the circulation, which would inevitably lead to higher levels of platelet activation. Whether or not there is a difference in the atherosclerotic plaque in the coronary and renal arteries is a subject that needs to be addressed in future research.

Despite compelling evidence from whole blood flow cytometry indicating that there is not a significant rise in platelet activation, thromboxane B2 levels increased significantly 24 hours after renal intervention. It is conceivable that whole blood flow cytometry may not be an adequate measure of platelet activation in patients undergoing renal artery revascularization. In light of
these conflicting results additional measures of platelet activation, such as serotonin levels, are required in order to determine whether platelet activation is associated with renal artery revascularization.

Interestingly, patients with renal dysfunction showed a significant rise in thromboxane B2 levels 24 hours after renal intervention. However, when patients with pre-existing renal dysfunction were excluded from the analysis, there was not a significant rise in thromboxane B2. It is possible that patients with pre-existing renal dysfunction are more prone to platelet activation. The role of thromboxane B2 in patients with renal dysfunction is a subject that needs to be addressed in future research trials.
CONCLUSIONS

Acute renal injury, defined as a rise in serum Cr of ≥ 0.5 mg/dl, occurs in approximately 7 to 14% of patients undergoing renal artery revascularization with endovascular stents. The presence of an un-repaired abdominal aortic aneurysm appears to increase this risk several fold, whereas a repaired aneurysm and other known risk factors for long-term renal dysfunction such as diabetes do not. Of concern, renal injury is associated with increased risk of subsequent renal failure, need for dialysis, and death. Whether renal injury can be prevented with embolic protection devices, and the impact this will have on late clinical events should be considered in future renal stent trials.

Despite compelling evidence from whole blood flow cytometry demonstrating that there is not a significant increase in platelet activation, we did see a significant rise in thromboxane B2 levels following renal artery revascularization. Additional measures of platelet activation are required in order to make definitive conclusions as to whether or not platelet activation is associated with renal artery revascularization.
References


in ostial atherosclerotic renovascular disease: a randomized trial. Lancet 353, 282-86.


Immunophenotypic Analysis of Platelets

With an average diameter of 3 μm, platelets are the smallest circulating cellular component in peripheral blood. The primary role of circulating platelets is to maintain hemostasis. The evaluation of platelets by flow cytometry has proven beneficial in the investigation of many disease states, including inherited defects such as Bernard-Soulier syndrome, Glanzmann thrombasthenia, and storage pool disease (Michelson et al., 2001). Flow cytometric techniques have been used in blood bank applications such as quality control of platelet concentrates, immunophenotyping of platelet surface receptor polymorphisms, platelet crossmatching, and detection of feto-maternal anti-platelet antibodies. Platelet hyporeactivity may result in potentially life-threatening bleeding including intracranial hemorrhage, while platelet hyperreactivity may result in intravascular thrombosis resulting in potentially life-threatening acute myocardial infarction or stroke. Consequently, antiplatelet therapies designed to reduce platelet responsiveness in vivo are now common practice in clinical cardiovascular medicine. Perhaps most common is the use of flow cytometry to study the role of platelet function and platelet activation in cardiovascular disease. A more complete list of the applications of flow cytometry to the study of platelets is shown in Table 6.10.1 and discussed in Michelson et al. (2001).

Resting platelets constitutively express many surface glycoproteins that are easily identified by flow cytometry. Upon platelet activation, many surface receptors are modulated in both copy number and conformation, while others, absent from the resting platelet surface, are newly expressed. This unit describes several strategies to evaluate platelet function by evaluating surface receptor expression on resting and activated platelets using flow cytometry. Three methods are described here in detail: determination of resting platelet surface receptor expression (see Basic Protocol 1 and Alternate Protocol); determination of platelet activation using P-selectin (CD62P) expression (see Basic Protocol 2), which reflects platelet α-granule release (McEver, 2001), or PAC1 binding, which detects the activated conformation of glycoprotein (GP) IIb-IIIa (integrin αmβ3; Shattil et al., 1985); and determination of procoagulant platelets and platelet-derived microparticles using annexin V binding or monoclonal antibodies specific for coagulation factors V/Va or X/Xa (see Basic Protocol 3; Furman et al., 2000). The methods described here are performed using the more physiologically relevant milieu of whole blood, which has the following advantages over platelet-rich plasma or washed platelet systems: (1) red cells and leukocytes are present, both of which affect platelet activation; (2) minimal sample manipulation minimizes artifactual in vitro activation and potential loss of platelet subpopulations; (3) both the activation state of circulating platelets and the reactivity of circulating platelets can be determined; (4) only minuscule volumes (~5 μl) of blood are required, making whole-blood flow cytometry particularly advantageous for neonatal studies; and (5) platelets of patients with profound thrombocytopenia can also be accurately analyzed. This unit does not specifically address platelet-associated IgG, heparin-induced thrombocytopenia, monitoring of GPIIb-IIIa receptor antagonists, reticulated platelets (see UNIT 7.10), leukocyte-platelet aggregate formation, or platelet counting. For a review of platelet-associated analysis techniques not covered by this unit see Schmitz et al. (1998).

Contributed by Lori A. Krueger, Marc R. Barnard, A.L. Freilinger III, Mark I. Furman, and Alan D. Michelson

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### Table 6.10.1 Applications of Flow Cytometry to the Study of Platelets

<table>
<thead>
<tr>
<th>Classification</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Measurement of platelet activation</strong></td>
<td>Activation-dependent monoclonal antibodies/reagents</td>
</tr>
<tr>
<td></td>
<td>Modulation of constitutively expressed surface receptors</td>
</tr>
<tr>
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<td>Procoagulant platelet-derived microparticles</td>
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<td>Leukocyte-platelet aggregates</td>
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<td></td>
<td>Platelet-platelet aggregates</td>
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<tr>
<td><strong>Diagnosis of specific disorders</strong></td>
<td>Bernard-Soulier syndrome</td>
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<td>Glanzmann thrombasthenia</td>
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<td>Immune thrombocytopenias</td>
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<td><strong>Monitoring of antiplatelet agents</strong></td>
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<td></td>
<td>Thienopyridines</td>
</tr>
<tr>
<td><strong>Monitoring of thrombopoiesis</strong></td>
<td>Reticulated platelets</td>
</tr>
<tr>
<td><strong>Blood bank applications</strong></td>
<td>Quality control of platelet concentrates</td>
</tr>
<tr>
<td></td>
<td>Identification of leukocyte contamination in platelet concentrates</td>
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<tr>
<td></td>
<td>Immunophenotyping of platelet HPA-1a</td>
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<tr>
<td></td>
<td>Detection of maternal and fetal anti-HPA-1a antibodies</td>
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<td></td>
<td>Platelet cross-matching</td>
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<tr>
<td><strong>Platelet counting</strong></td>
<td></td>
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<tr>
<td><strong>Research applications</strong></td>
<td>Platelet survival, tracking, and function in vivo</td>
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<td></td>
<td>Platelet recruitment</td>
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<tr>
<td></td>
<td>Bacteria-platelet interactions</td>
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<tr>
<td></td>
<td>Calcium flux</td>
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<td></td>
<td>Cytoskeletal rearrangement</td>
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<tr>
<td></td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td></td>
<td>Signal transduction</td>
</tr>
</tbody>
</table>

*Table order reflects the most commonly studied, and relevant applications. Includes circulating activated platelets, platelet hyperreactivity, or platelet hyporeactivity.

### STRATEGIC PLANNING

#### Blood Collection

Careful experimental planning is required for accurate and consistent results when immunophenotyping platelets by flow cytometry. To minimize ex vivo platelet activation, blood samples should be processed within ~30 min after drawing blood for many assays. The act of drawing blood is itself a potential source of artifactual platelet activation; therefore, the following recommendations are suggested (Michelson et al., 2001):

- Use a light tourniquet or none at all
- Use a 21-G (or larger bore) needle
- Ensure a smooth draw (i.e., good flow)
- Discard the first 2 ml of blood drawn

Adhering to these recommendations will minimize tissue thromboplastin contamination of blood samples and red cell hemolysis that could lead to artifactual platelet activation.
Table 6.10.2 Anticoagulants Used in the Study of Platelets

<table>
<thead>
<tr>
<th>Anticoagulant</th>
<th>Mechanism of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid citrate dextrose (ACD)</td>
<td>Weak Ca(^{2+}) chelator</td>
</tr>
<tr>
<td>Citrate theophylline adenosine dipyridimole (CTAD)</td>
<td>Chelates Ca(^{2+}) and increases intracellular cAMP, keeping platelets &quot;quiet&quot;</td>
</tr>
<tr>
<td>Corn trypsin inhibitor</td>
<td>Activated coagulation factor XII inhibitor</td>
</tr>
<tr>
<td>EDTA(^{-})</td>
<td>Strong Ca(^{2+}) chelator, dissociates GPIIb-IIIa complex</td>
</tr>
<tr>
<td>Heparin(^{+})</td>
<td>Combines with anti-thrombin III to inhibit thrombin activity</td>
</tr>
<tr>
<td>Hirudin</td>
<td>Direct thrombin inhibitor</td>
</tr>
<tr>
<td>D-Phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (P-PACK)</td>
<td>Direct thrombin inhibitor</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>Weak Ca(^{2+}) chelator</td>
</tr>
</tbody>
</table>

\(^{+}\)These anticoagulants should be avoided for evaluation of platelet function studies by flow cytometry (see Strategic Planning).

Each laboratory should determine whether their method of collection, including the drawing of samples through angioplasty and other catheters, results in artifactual in vitro platelet activation, as determined by the binding of activation-dependent monoclonal antibodies.

**Choice of Anticoagulant**

Although sodium citrate (a weak calcium chelator) is the most common anticoagulant used for platelet studies, others have been successfully used. EDTA (a strong calcium chelator) should be avoided, because it causes dissociation of the integrin \(\alpha_{\text{IIb}}\beta_3\) (GPIIb-IIIa) complex. Heparin should also be avoided because it binds to, and may activate, platelets. Nonchelating anticoagulants such as P-PACK (a direct thrombin inhibitor) may be preferable for the monitoring of GPIIb-IIIa antagonist therapy. The anticoagulants listed in Table 6.10.2 have reportedly been used in studies of platelets.

**Sample Handling**

The length of time between sample draw and sample preparation should be minimized to reduce spontaneous platelet activation. The blood should be properly mixed with anticoagulant, avoiding unnecessary agitation prior to testing. The whole-blood samples should be collected and maintained in a container with nonwettable surfaces such as siliconized glass or polypropylene.

**Experimental Design**

Many platelet surface receptors are modulated during platelet activation (see Table 6.10.3). For example, GPIb-IX-V may be cleaved and/or internalized to the surface-connected canalicular system upon platelet activation (Michelson et al., 1996a). This must be taken into consideration in experimental design. For example, if using a GPIIb\(\alpha\)-specific antibody (i.e., anti-CD42b) as a platelet identifier, then an adjustment in the instrument threshold (or discriminator) may be necessary when evaluating activated versus resting platelets. Alternatively, if the reduction in platelet GPIIb\(\alpha\) is being used as an indicator of platelet activation, then the maximal change in receptor expression will be observed only if platelets are labeled with the GPIIb\(\alpha\)-specific antibody after platelet activation has taken place. If a directly-conjugated GPIb-IX-specific test monoclonal antibody is added to the platelets prior to activation, then the activation-induced redistribution of GPIb-IX to the surface-connected canalicular system will not result in a significant decrease in platelet fluorescence, because fluorescence will be detected irrespective of whether the conjugated antibody is on the surface of or within the platelet; therefore, in flow cytometric

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Phenotypic Analysis

6.10.3

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### Table 6.10.3 Activation-Dependent Changes in Platelet Surface Labeling of Monoclonal Antibodies and Annexin V

<table>
<thead>
<tr>
<th>Changes in surface receptor expression</th>
<th>Resting platelet</th>
<th>Activated platelet</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD36</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>GPIb-IX</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>GPIIb-IIIa</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Conformational changes in GPIIb-IIIa (integrin αIIbβ3)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ligand-induced binding sites (LIBS)</td>
<td>–</td>
<td>+++</td>
</tr>
<tr>
<td>PAC1</td>
<td>–</td>
<td>+++</td>
</tr>
<tr>
<td>Receptor-induced binding sites on fibrinogen (RIBS)</td>
<td>–</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Development of a procoagulant surface</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Factor VIII binding</td>
<td>–</td>
<td>+++</td>
</tr>
<tr>
<td>Factor V/Va binding</td>
<td>–</td>
<td>+++</td>
</tr>
<tr>
<td>Factor X/Va binding</td>
<td>–</td>
<td>+++</td>
</tr>
<tr>
<td>Phosphatidylserine expression (detected by annexin V)</td>
<td>–</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Exposure of granule membrane proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD40L (or CD154)</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>CD63 (lysosomes)</td>
<td>–</td>
<td>++</td>
</tr>
<tr>
<td>LAMP-1 (lysosomes)</td>
<td>–</td>
<td>++</td>
</tr>
<tr>
<td>LAMP-2 (lysosomes)</td>
<td>–</td>
<td>++</td>
</tr>
<tr>
<td>Lectin-like oxidized LDL receptor-1 (LOX-1)</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>P-selectin (CD62P, α-granules)</td>
<td>–</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Platelet surface binding of secreted platelet proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multimerin</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Thrombospondin</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

*Annexin V is a 35 to 36 kDa protein that binds to phosphatidylserine in the presence of Ca²⁺.*

assays examining platelet surface GPIb-IX modulation, GPIb-IX-specific antibodies that are directly conjugated must be added to the assay after the addition of the agonist.

**IMMUNOPHENOTYPING OF PLATELET SURFACE RECEPTORS**

This procedure may be used to qualitatively and/or quantitatively evaluate a platelet surface receptor such as GPIIb-IIIa (reduced or absent in Glanzmann thrombasthenia) or GPIb-IX-V (reduced or absent in Bernard-Soulier syndrome). Additionally, this protocol may be used to evaluate the in vivo activation status of circulating platelets.

The optimal final concentration of platelet-specific monoclonal antibody reagents must be determined by titration. At a minimum, two antibodies, each conjugated to a different fluorochrome (e.g., fluorescein and phycoerythrin), are used. The fluorescent conjugate of the platelet identifier determines the thresholding parameter used in the flow cytometric analysis. The choice of the platelet identifier is determined by the analysis being performed. For example, anti-CD42a or anti-CD42b (GPIIX- and GPIbα-specific, respectively) are used as platelet identifiers when measuring surface expression of GPIIb-IIIa (CD41 and CD61) in the investigation of Glanzmann thrombasthenia, an inherited deficiency of GPIIb-IIIa. Anti-CD41 or anti-CD61 may be used as platelet identifiers when investigating Bernard-Soulier syndrome, an inherited deficiency of GPIb-IX-V (CD42b, CD42a, and CD42d). Anti-CD41, -CD61, -CD42a, or -CD42b may be used as platelet identifiers when evaluating activation-dependent receptors such as P-selectin.
(recognized by anti-CD62P) and PAC1; however, surface expression of GPIIb-IIIa and GPIb-IX is modulated upon platelet activation, which must be considered when selecting a platelet identifier. Monoclonal antibody reagents are prepared in modified HEPES/Tyrode’s (HT) buffer.

**Materials**

Whole blood (WB) containing anticoagulant (see Strategic Planning) or isolated platelets (see Support Protocol)
Modified HT buffer (see recipe)
Platelet-specific antibody cocktail titrated in modified HT buffer (minimum two antibody specificities each conjugated to a different fluorochrome):
Specific platelet identifier: monoclonal anti-CD41, -CD61, -CD42a, or -CD42b
Marker of platelet activation: monoclonal anti-CD62P or PAC1
Negative control: antibody isotype-, concentration-, fluorochrome-, and F:P ratio-matched to the activation marker, or blocking agent inhibiting platelet-specific marker binding
1% formalin fixative (see recipe)

1. Within 30 min of blood draw, dilute whole blood (WB) containing anticoagulant 1/10 in modified HT buffer (e.g., 10 μl WB and 90 μl HT).

   *In the diagnosis of Glanzmann thrombasthenia and Bernard-Soulier syndrome it is wise to use blood from a normal healthy donor analyzed in parallel with patient blood to differentiate normal and abnormal expression of GPIIb-IIIa or GPIb-IX, respectively.*

2. Immediately mix diluted whole blood (dWB) with appropriately titrated platelet-specific antibody cocktail (e.g., 10 μl dWB and 30 μl antibody cocktail). Include a negative control if a platelet activation marker is used. Incubate at room temperature (e.g., 20 min).

   *Individual laboratories must determine the optimal antibody concentration and incubation time.*

3. Fix labeled cells with 10× to 20× total assay volume (e.g., 400 to 800 μl) of 1% formalin solution. Let fixation proceed 15 min at room temperature, then place tubes at 4°C until analysis.

   *Stability of fixed preparations must be determined by individual laboratories.*

4. Analyze by flow cytometry using the following setup:

   a. Use the platelet identifier as the thresholding (or discriminating) parameter (Fig. 6.10.1A).

   b. Collect light-scatter parameters in logarithmic mode. (Heterogeneity in platelet size and platelet morphology contributes to a relatively broad distribution of light scattering detectable by flow cytometry.)

   c. Analyze diluted fixed sample using low flow rate (e.g., 150 to 250 platelet events per second) to minimize, as far as possible, coincidence between two or more events.

   d. Identify single platelet events by their characteristic light scatter and positive labeling with platelet-specific identifier (Fig. 6.10.1B).

   e. Measure the platelet surface receptor or receptors of interest from gated events (Fig. 6.10.1C).

   *Platelets are smaller than lymphocytes and erythrocytes; therefore, high-voltage settings used for leukocyte and erythrocyte studies may not be appropriate for platelet analysis.*

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<table>
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<th>6.10.5</th>
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<tr>
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</table>
Figure 6.10.1 Evaluation of platelet activation by whole-blood flow cytometry. (A) Platelets labeled with specific platelet identifier. The instrument threshold (or discriminator) is set on the fluorescence parameter corresponding to the conjugate of the platelet identifier. (B) Characteristic light-scatter profile of a platelet population in diluted whole blood. Data are collected and displayed using logarithmic-orthogonal and logarithmic-forward light scatter. Single platelet events are identified by their characteristic light-scatter properties and positive labeling with a platelet-specific monoclonal antibody reagent. (C) Single-parameter fluorescence histogram of the platelet activation marker P-selectin (CD62P). The positive analysis region is determined by the negative isotype control (thin solid line). Events displayed are generated from gated events in both panel A and panel B. (D) Determination of procoagulant platelets and platelet-derived microparticles. Two-parameter histogram displaying Factor Xa binding versus forward-angle light scatter. The analysis region for platelets capable of binding coagulation protein—activated factors V and X or expressing phosphatidylinerine is established on this two-parameter histogram.

ImmunoPhenotyping of Platelet Surface Receptors

Whole blood may be fixed first and subsequently labeled with platelet-specific reagents. Many platelet surface receptors are well preserved and recognized by specific monoclonal antibodies after formalin fixation; however, labeling intensity may diminish with prolonged fixation. Antibody concentrations required for optimal labeling may be different for fixed versus unfixed cells; therefore, optimal antibody concentrations should be verified by titration using fixed cells. Some antigen epitopes such as that recognized by PAC1 may not label after formalin fixation.
**Additional Materials** (also see Basic Protocol 1)

2% formalin fixative (see recipe)

1. Within 30 min of blood draw, dilute WB 1:1 with 2% formalin fixative. (i.e., 100 µl WB and 100 µl of 2% formalin). Fix 15 to 60 min at room temperature. Store samples at 4°C.

   *Antibody labeling may decrease in intensity with prolonged fixation; therefore, the length of time that fixed samples can be stored prior to labeling must be determined by individual laboratories for each antibody clone being used.*

2. Dilute fixed WB 1:10 in modified HT buffer.

   *Dilution can be performed prior to storage at 4°C.*

3. Mix dWB with appropriately titrated antibody cocktail (e.g., 20 µl dWB and 20 µl antibody cocktail) and incubate at room temperature (e.g., 20 min).

   *Individual laboratories must determine the optimal antibody concentration and incubation time.*

4. Dilute labeled cells with 10× to 20× total assay volume (e.g., 400 to 800 µl) of 1% formalin solution. Place tubes at 4°C until analysis.

   *Stability of fixed preparations must be determined by individual laboratories.*

5. Analyze by flow cytometry as described above (see Basic Protocol 1, step 4)

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**DETERMINATION OF PLATELET ACTIVATION USING P-SELECTIN OR PAC1 EXPRESSION**

This procedure may be used to evaluate platelet reactivity in response to agonist.

**Materials**

Whole blood (WB) containing anticoagulant (see Strategic Planning) or isolated platelets (see Support Protocol)

Modified HT buffer (see recipe)

Platelet-specific antibody cocktail titrated in modified HT buffer (minimum two antibody specificities each conjugated to a different fluorochrome): Specific platelet identifier: e.g., monoclonal anti-CD41, -CD61, -CD42a, -CD42b

Specific marker of platelet activation: e.g., monoclonal anti-CD62P or -PAC1

Platelet agonist (see recipe): e.g., ADP, epinephrine, human α-thrombin, thrombin receptor-activating peptide (TRAP)

Negative controls: antibody isotype-, concentration-, fluorochrome-, and F:P ratio-matched to the specific activation marker, or blocking agent that inhibits the platelet-specific marker binding

10 mM GPRP (see recipe)

1% formalin fixative (see recipe)

1. Within 30 min of blood draw, dilute WB 1:10 in modified HT buffer (i.e., 10 µl WB and 90 µl HT).

2. Immediately mix dWB with appropriately titrated platelet-specific antibody cocktail and with platelet agonist (e.g., 10 µl dWB, 20 µl antibody cocktail, and 10 µl platelet agonist). Include a negative control if a platelet activation marker is used. Incubate at room temperature (e.g., 20 min). If thrombin is being used as the platelet agonist, add GPRP to a final concentration of 2.5 µM to prevent fibrin polymerization and clot formation (Michelson, 1994).
The fluorescent conjugate of the platelet identifier determines the thresholding parameter used in the flow cytometric analysis. Surface expression of GPIIb-IIIa and GPIb-IX are modulated upon platelet activation, which must be considered when selecting a platelet identifier, establishing instrument thresholds, and gating platelet populations.

Individual laboratories must determine the optimal antibody concentration and incubation time.

3. Fix labeled cells with 10× to 20× total assay volume (e.g., 400 to 800 μl) of 1% formalin fixative. Let fixation proceed 15 min at room temperature, then place tubes at 4°C until analysis.

Stability of fixed preparations must be determined by individual laboratories.

4. Analyze by flow cytometry as described above (see Basic Protocol 1, step 4).

**Determination of procoagulant platelets using Annexin V binding or monoclonal antibodies specific for coagulation factor V/Va or X/Xa**

This procedure may be used to evaluate procoagulant platelets and the ability of platelets to generate procoagulant microparticles in response to an agonist (Furman et al., 2000).

**Materials**

Whole blood (WB) containing anticoagulant (see Strategic Planning) or isolated platelets (see Support Protocol)

Modified HT buffer (see recipe) containing 5 mM GPRP (see recipe)

Coagulation factor V/Va or X/Xa

Platelet agonist (see recipe) supplemented with 6 mM CaCl₂; e.g., collagen, combined thrombin/collagen mixture, or calcium ionophore A23183

Modified HT buffer

Platelet-specific antibody cocktail titrated in modified HT buffer—minimum two antibodies, or one identifier and annexin V—each conjugated to a different fluorochrome:

Specific platelet identifier: e.g., anti-CD41, anti-CD61, anti-CD42a, or anti-CD42b

Marker of platelet procoagulant activity: e.g., annexin V, monoclonal anti-coagulation factor V/Va or X/Xa

1% formalin fixative (see recipe)

1. Within 1 hr of draw, dilute WB 1:10 in modified HT buffer containing 5 mM GPRP (i.e., 10 μl WB and 90 μl HT/GPRP). If coagulation factor V/Va or X/Xa is to be detected, include these factors in the HT/GPRP diluent.

Sources of V/Va or X/Xa include autologous platelet-poor plasma or purified coagulation factors.

Each individual laboratory must titrate the optimal concentration of autologous plasma or purified coagulation factor to add back for optimal platelet surface detection.

2. Immediately combine dWB with an equal volume of platelet agonist supplemented with 6 mM CaCl₂, or modified HT buffer alone—e.g., 15 μl dWB/GPRP and 15 μl agonist or buffer alone (negative control). Incubate 20 min at 37°C.

The optimal incubation time must be determined by individual laboratories.

The final concentration of GPRP is 2.5 mM during the assay incubation; therefore, for a 1:1 (dWB:agonist) mix in the assay, the GPRP concentration in the dWB buffer is 2× or 5 mM. Similarly, HT supplemented with 3 mM Ca²⁺ (final concentration) used alone or as the agonist diluent is prepared 2× or 6 mM.
3. Label with titrated platelet-specific antibody cocktail (e.g., 30 μl dWB/GPRP/agonist or buffer, and 10 μl antibody reagent cocktail) 20 min at room temperature.

As a minimum, two antibody specificities (or one platelet identifier and annexin V) each conjugated to a different fluorochrome (such as fluorescein and phycoerythrin) are used. The fluorescent conjugate of the platelet identifier determines the thresholding (or discriminating) parameter used in the flow cytometric analysis. Surface expression of GPIb-IIIa and GPIb-IX is modulated upon platelet activation, which must be considered when selecting a platelet identifier. The optimal final concentration of platelet-specific monoclonal antibody reagents must be determined by titration.

4. Fix labeled cells 15 min with 10× to 20× total assay volume (e.g., 400 to 800 μl) of 1% formalin fixative at room temperature. Place tubes at 4°C until analysis. Stability of fixed preparation must be determined by individual laboratories.

5. Analyze by flow cytometry as described (see Basic Protocol 1, step 4).

Procoagulant platelets often display dramatic light-scatter changes requiring adjustments to fluorescence and to light scatter gates. Additionally, the distinction between the procoagulant (positive labeling for annexin V, anti-V/Na, or anti-X/Xa) and nonprocoagulant (negative labeling-for annexin V, anti-V/Na, or anti-X/Xa) phenotype may be difficult to define on a single-parameter fluorescence histogram; therefore, a two-parameter histogram displaying fluorescence (annexin V, anti-V/Na, or anti-X/Xa) versus forward-angle light scatter may better define the procoagulant platelet population (Fig. 6.10.1, panel D).

## PREPARATION OF ISOLATED PLATELETS

Some studies may require the isolation of platelets from other cellular and/or plasma components. Platelets may be separated from other cellular components by centrifugation and further isolated from plasma components by gel filtration or washing.

### Materials

- Anticoagulated whole blood collected in 5-ml vacutainer tubes or 15-ml conical tubes if drawn by syringe
- Sepharose 2B beads
- Modified HT buffer (see recipe)
- Citrate wash buffer (see recipe)
- Benchtop centrifuge with rotors for 5-ml Vacutainer tubes and 15-ml conical tubes
- Polypropylene or siliconized glass test tube
- 10-ml syringe column
- 15-ml conical tubes

### Preparation of platelet-rich plasma

1. Within 30 min of blood draw, prepare platelet-rich plasma (PRP) by centrifuging anticoagulated whole blood collected in 5-ml vacutainer tubes or 15-ml conical tubes if drawn by syringe in a benchtop centrifuge with an appropriate rotor 10 to 15 min at 150 to 200 × g, room temperature. Remove PRP to a clean polypropylene or siliconized glass test tube, being careful not to disturb the buffy coat and red cell layers (also see UNIT 5.1).
Isolation of platelets by gel filtration:
2a. Pack a 10-ml syringe column with Sepharose 2B beads.
3a. Equilibrate column with 10 vol deionized water, followed by 3 vol modified HT buffer.
4a. Layer PRP (step 1) on the top of the column.
5a. Let PRP fully enter the column and carefully layer with 30 ml modified HT.
6a. Collect gel-filtered platelets (i.e., eluent).

Isolation of platelets by centrifugation:
2b. Fill a 15-ml conical tube containing 1 to 8 ml PRP (step 1) with citrate wash buffer.
3b. Centrifuge in a benchtop centrifuge with an appropriate rotor 10 min at 1,200 × g, at room temperature.
4b. Aspirate the supernatant and gently resuspend platelet pellet.
5b. Repeat wash procedure (steps 2b to 4b).
6b. After final wash, resuspend platelet pellet in modified HT.

Use isolated platelets as soon as possible after preparation, regardless of preparation technique. Isolated platelet preparations may be substituted for whole blood in any of the preceding basic protocols.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. Pass all reagents through 0.2- to 0.4-μm filters prior to use. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Citrate wash buffer
11 mM glucose
128 mM NaCl
4.3 mM NaH₂PO₄
7.5 mM Na₂HPO₄
4.8 mM sodium citrate
2.4 mM citric acid
0.35% (w/v) BSA
Adjust pH to 6.5 with 0.1 M NaOH or 0.1 M HCl
Store up to 1 year at -20°C.
Bring to room temperature and add 1 mg/ml prostaglandin PGE₁ (see recipe) to a final concentration of 50 ng/ml immediately prior to use.

Formalin fixative, 1%, 2%
Dilute 10% (v/v) ultrapure methanol-free formalin (Polysciences) to 1% or 2% (v/v) in HEPES buffered saline (HBS; see recipe). Store up to 1 month at 4°C. Bring to room temperature prior to use.

GPRP, 10 mM
Dilute GPRP (Gly-Pro-Arg-Pro) in modified HT buffer (see recipe) to a concentration of 10 mM. Store up to 1 week at 4°C or 1 year at -20°C. Bring to room temperature prior to use.

Use for diluting thrombin and in assays containing CaCl₂ to prevent fibrin polymerization and clot formation (Michelson, 1994).
**HEPES buffered saline (HBS)**

10 mM HEPES
0.15 mM NaCl
Adjust pH to 7.4 with 0.1 M NaOH or 0.1 M HCl
Store up to 6 months at 4°C
Bring to room temperature prior to use

**Modified HEPES/Tyrode's (HT) buffer**

10 mM HEPES
137 mM NaCl
2.8 mM KCl
1 mM MgCl₂
12 mM NaHCO₃
0.4 mM Na₂HPO₄
0.35% (w/v) BSA
5.5 mM glucose
Adjust pH to 7.4 with 0.1 M NaOH or 0.1 M HCl
Store up to 1 week at 4°C or 1 year at −20°C
Bring to room temperature prior to use

*pH may need readjustment after storage at 4°C.*

**Prostaglandin PGE₁**

Prepare 1 mg/ml stock solution in 100% ethanol. Store in small aliquots at −80°C until immediately prior to use. Avoid multiple freeze/thaw cycles.

**Platelet agonists**

Prepare all platelet agonist working solutions (concentrations determined empirically) by diluting stocks in modified HT buffer (see recipe), with or without 6 mM CaCl₂ as appropriate, just prior to use. Bring to room temperature just before use. Discard left-over working solutions daily.

*ADP*: Adenosine diphosphate (ADP) is typically used at concentrations of 20 μM (maximal platelet activation in diluted whole blood) to submaximal doses of 0.5 μM. Store stock ADP according to manufacturer’s instructions (often frozen at −20°C).

*Calcium ionophore A23187*: Prepare 10 mM stock A23187 in DMSO and store up to 1 year at −80°C. A23187 is typically used at concentrations of 10 to 20 μM (maximal platelet activation in diluted whole blood). Dilute stock at least 1:100 (final DMSO concentration <1%) in modified HT buffer (see recipe) supplemented with 6 mM CaCl₂. Use in the evaluation of procoagulant platelets and platelet-derived microparticles.

*Collagen*: Collagen is typically used at concentrations of 10 to 20 μg/ml alone or in combination with submaximal doses of thrombin (see below). Dilute in modified HT buffer (see recipe) supplemented with 6 mM (2×) CaCl₂. Use in the evaluation of procoagulant platelets and platelet-derived microparticles in the presence of 2.5 mM (final concentration) GPRP (see recipe). Store stock collagen according to manufacturer’s instructions.

*Epinephrine*: Epinephrine is often used in combination with ADP (see above) at concentrations of 10 to 20 μM (maximal platelet activation in diluted whole blood). Store stock epinephrine according to manufacturer’s instructions.

*continued*
**Human α-thrombin:** Thrombin is typically used at concentrations of 1 to 2 U/ml (maximal platelet activation in diluted whole blood) to submaximal doses of 0.1 U/ml. The concentration of thrombin required to achieve maximal platelet activation in plasma-free systems is ~0.1 U/ml. Dilute working concentration of thrombin in modified HT buffer (see recipe) containing sufficient 10 mM GPRP (see recipe) to give a final assay concentration of 2.5 mM. Store 200 U/ml stock concentration up to 6 months at −80°C or up to one week at 4°C.

**Thrombin receptor-activating peptide (TRAP/SFLLRN):** TRAP is typically used at concentrations of 20 to 50 μM (maximal platelet activation in diluted whole blood) to submaximal doses of 1.5 to 5 μM. Dilute SFLLRN (Ser-Phe-Leu-Leu-Arg-Asp) in modified HT buffer (see recipe). Store up to one week at 4°C or 1 year at −80°C.

**COMMENTARY**

**Background Information**

The use of flow cytometry for the study of platelet function and platelet activation encompasses multiple assays for multiple purposes (Table 6.10.1). While this unit does not describe procedures for all the assays outlined in Table 6.10.1, many facets of platelet function can be evaluated using the protocols described herein.

In the absence of an exogenously added platelet agonist (see Basic Protocol 1), the activation state of circulating platelets in vivo, as judged by the binding of an activation-dependent monoclonal antibody or similar reagent, can be determined. Circulating activated platelets have been detected in patients with stable and unstable angina, acute myocardial infarction, acute cerebrovascular ischemia, peripheral arterial occlusive disease, diabetes mellitus, pre-eclampsia, hemodialysis, systemic inflammatory response syndrome, septic multiple organ dysfunction syndrome, myeloproliferative disorders, and Alzheimer disease. Platelet-derived microparticles are increased in acute coronary syndromes, cardiopulmonary bypass, transient ischemic attacks, and patients with prosthetic heart valves. Platelet hyporeactivity has been reported in very-low-birth-weight preterm neonates and may contribute to the propensity of intraventricular hemorrhage in that patient group.

Bernard-Soulier syndrome is an inherited deficiency of the GPIb-IX-V complex. Flow cytometric analysis with GPIb-, GPIIX-, and GPV-specific monoclonal antibodies (i.e., anti-CD42b, -CD42a, and -CD42d, respectively) provides a rapid and simple means for the diagnosis of the homozygous and heterozygous states of Bernard-Soulier syndrome (Michelson, 1987). Whole-blood flow cytometry allows analysis of platelets without attempting the technically difficult procedure of physically separating the giant Bernard-Soulier syndrome platelets from similarly sized red and white blood cells. Because light scatter (especially forward light scatter) correlates with platelet size, light-scatter gates may need to be adjusted in the flow cytometric analysis of giant platelet syndromes such as Bernard-Soulier syndrome. This adjustment may result in overlap of the light scatter of giant platelets with red and white blood cells. It is therefore essential to include in the assay a platelet-specific monoclonal antibody as a platelet identifier. For Bernard-Soulier syndrome platelets, this identifier antibody obviously cannot be GPIIb, GPIIX, or GPV specific.

Glanzmann thrombasthenia is an inherited deficiency of integrin αIIbβ3 (GPIIb-IIIa). Flow cytometric analysis with anti-CD41- and anti-CD61-specific monoclonal antibodies provides a rapid and simple means for the diagnosis of the homozygous and heterozygous states of Glanzmann thrombasthenia (Jennings et al., 1986). In addition, a panel of activation-dependent monoclonal antibodies can be used to evaluate patients with defects in platelet aggregation, secretion, or procoagulant activity.

The inclusion of an agonist in the assay enables evaluation of the reactivity of circulating platelets in vitro (see Basic Protocol 2). Thus, whole-blood platelet flow cytometry may be used as a physiological assay of platelet function in that a platelet agonist results in a specific functional response (i.e., a change in the surface expression of a physiological receptor or bound ligand) as determined by the binding characteristics of a monoclonal antibody or similar reagent.

**Activation-dependent monoclonal antibodies**

Laboratory markers of platelet activation include activation-dependent conformational changes in the GPIIb-IIIa complex (integrin αIIbβ3, CD41/CD61), exposure of granule
membrane proteins, platelet surface binding of secreted platelet proteins, and development of a procoagulant surface (Table 6.10.2). The two most widely studied types of activation-dependent monoclonal antibodies are those directed against granule membrane proteins and those directed against conformational changes in GPIIb-IIIa.

P-selectin is a component of the α-granule membrane of resting platelets that is expressed on the platelet surface membrane only after α-granule secretion, which occurs during platelet activation (McEver, 2001). Thus, a P-selectin-specific monoclonal antibody binds only to degranulated platelets, not to resting platelets. The activation-dependent increase in platelet surface P-selectin is not reversible over time in vitro; however, in vivo circulating degranulated platelets rapidly lose their surface P-selectin, but continue to circulate and function (Michelson et al., 1996b). Platelet surface P-selectin is therefore not an ideal marker for the detection of circulating degranulated platelets, unless the blood sample is drawn immediately distal to the site of platelet activation, the blood sample is drawn within 5 min of the activating stimulus, or there is continuous activation of platelets. The length of time that other activation-dependent surface markers remain expressed on the platelet surface in vivo has not yet been definitively determined.

GPIIb-IIIa is a receptor for fibrinogen and von Willebrand factor that is essential for platelet aggregation. Whereas most monoclonal antibodies directed against GPIIb-IIIa (anti-CD41 and -CD61) bind to resting platelets, monoclonal antibody PAC1 is directed against the fibrinogen binding site exposed by a conformational change in GPIIb-IIIa (Shatil et al., 1985). Thus, PAC1 binds only to activated platelets, not to resting platelets. Other GPIIb-IIIa-specific activation-dependent monoclonal antibodies are directed against either ligand-induced conformational changes in GPIIb-IIIa—ligand-induced binding sites (LIBS; Frelinger et al., 1988)—or receptor-induced conformational changes in the bound ligand (i.e., fibrinogen)—receptor-induced binding sites (RIBS; Zamarron et al., 1990; Table 6.10.3). Rather than GPIIb-IIIa-specific monoclonal antibodies, fluorescein-conjugated fibrinogen can also be used in flow cytometric assays to detect the activated form of platelet surface GPIIb-IIIa (Faraday et al., 1994), but the concentration of unlabeled plasma fibrinogen and unlabeled fibrinogen released from platelet α granules must also be considered in these assays.

As determined by flow cytometry, in vitro activation of platelets by some agonists (e.g., collagen, collagen/thrombin, C5b-9, calcium ionophore A23187) in the presence of extracellular calcium ions results in platelet-derived microparticles (defined by low forward-angle light scatter and binding of a platelet-specific monoclonal antibody; see Basic Protocol 3) that are procoagulant (determined by binding of monoclonal antibodies to activated factors V, X, or VIII, or by annexin V; Gilbert et al., 1991; Holme et al., 1995; Furman et al., 2000). These findings suggest that procoagulant platelet-derived microparticles may have an important role in the assembly of the "tenase" and "prothrombinase" components of the coagulation system in vivo. A flow cytometric method for the direct detection of procoagulant platelet-derived microparticles in whole blood has been developed (Rajasekhar et al., 1993).

Critical Parameters and Troubleshooting

Minimizing platelet aggregates

Platelet aggregates can be minimized in the preparation of platelets for whole-blood flow cytometry by following these recommendations (Michelson et al., 2001):

1. Prepare reagents in advance and avoid delays in procedure.
2. Use appropriate blood collection technique (see Strategic Planning).
3. Use polypropylene (or siliconized glass) tubes and syringes.
4. Mix blood immediately with the anticoagulant.
5. Do not include washing, centrifugation, gel filtration, vortexing, or stirring steps.
6. Reduce the platelet count by diluting the samples.
7. Include the synthetic tetrapeptide GPRP in the assay if thrombin is the agonist.
8. Mix gently after addition of agonist, then incubate undisturbed.

Arg-Gly-Asp (RGD)-containing peptides have also been used to minimize platelet aggregates, but these peptides may interfere with the binding of detecting antibodies (e.g., PAC1) and result in exposure of LIBS.

Each sample should be monitored for evidence of platelet aggregation—i.e., "smearing" of the platelets into the upper right quadrant of the log side (orthogonal) light scatter versus log forward light scatter histogram.
Platelet activation by thrombin, one of the most physiologically important platelet activators, can be directly measured in whole blood through the use of GPRP (Michelson, 1994). In the absence of GPRP, addition of thrombin to whole blood results in a fibrin clot, thereby precluding the use of thrombin as an agonist in the whole-blood assay. Furthermore, thrombin is a potent inducer of platelet-to-platelet aggregation, which precludes analysis by flow cytometry of activation-dependent changes in individual platelets; however, addition to whole blood of GPRP together with thrombin inhibits both fibrin polymerization and, to a lesser extent, platelet-to-platelet aggregation, without affecting thrombin-induced platelet activation. An alternative to the use of thrombin and GPRP in a whole-blood flow cytometric assay is the use of TRAP, a peptide fragment of the protease-activated receptor 1 (PAR1) “tethered ligand” receptor for thrombin (Vu et al., 1991). Without the need for GPRP, TRAP directly activates platelets in whole blood without resulting in a fibrin clot; however, TRAP may not reflect all aspects of thrombin-induced platelet activation, because PAR1 is not the only platelet receptor for thrombin. TRAP is also useful in patient populations where therapeutic heparin (a direct thrombin inhibitor) has been administered.

Whole-blood flow cytometric assays frequently employ a GPIb- or GPIIX-specific monoclonal antibody to identify platelets. The activation-induced decrease in the platelet surface expression of GPIb-IX generally does not result in fluorescence below the threshold used to distinguish platelets from other cells and debris. Thus, no subpopulations of platelets are excluded. A method of avoiding the activation-induced decrease in binding of a GPIb-specific monoclonal antibody is to add a direct conjugate of the GPIb-specific antibody before addition of the agonist.

To specifically analyze the activation-induced decrease in the platelet surface expression of the GPIb-IX complex in whole blood, a CD41- or CD61-specific monoclonal antibody can be employed as the platelet-identifying reagent and the GPIb-IX specific reagent should be added after platelet activation has occurred (i.e., after the addition of agonist).

Lag time

Perhaps the single biggest obstacle to the widespread use of flow cytometry in the study of platelet activation is the inconvenience of initiating testing within 30 min of drawing blood. To circumvent this problem, platelets may be fixed first with 1% formalin prior to labeling with monoclonal antibody reagents. Fix-first methods do not lead to artifactual platelet activation in the authors’ hands. The biggest disadvantages are that some activation-dependent epitopes are no longer recognized by their specific monoclonal reagents and that fix-first methods preclude the ability to study platelet responsiveness to agonists.

Other elapsed-time considerations are between: fix-first and labeling; fixation after labeling and analysis; and use of PRP or purified platelets instead of whole blood. There is generally a time-dependent decrease in the ability to label fixed platelets. Additionally, the fluorescence intensity after labeling and subsequent fixation generally decreases in a time-dependent manner; therefore, the stability of fixed samples prior to flow cytometric analysis must be determined by individual laboratories.

**Fixation**

Sample fixation is advantageous in a clinical setting where there may not be immediate access to a flow cytometer. Fixation prevents subsequent artifactual in vitro platelet activation. For most antibodies, the “antibody labeling before fixation” method described above results in no significant differences in fluorescence intensity between samples analyzed immediately and samples analyzed within 24 hr of fixation. A “fixation before antibody labeling” method also results in no significant differences in fluorescence intensity between samples analyzed immediately and samples analyzed within 24 hr of antibody labeling; however, fixation is an important variable to be controlled for, especially in a “fixation before antibody labeling” method, because the binding of activation-dependent monoclonal antibodies to fixed platelets is often decreased compared to that of unfixed platelets (Michelson et al., 1995). Furthermore, the binding of some antibodies further decreases after fixation in a time-dependent manner. The optimal fixation method for each new monoclonal antibody must therefore be defined by each laboratory.

A compelling argument in favor of immediate sample fixation is that activation-dependent changes are often time-dependent, at least in vitro. For example, the platelet surface expression of the GPIb-IX-V complex decreases within 30 sec of platelet activation, reaching a nadir at ~5 min, but over the next ~45 min the platelet surface expression of the GPIb-IX-V complex returns to normal (Michelson et al., 1996).
1996a). The activation-dependent increase in the platelet surface expression of GPIIb-IIIa and CD40 ligand (CD40L, CD154) is also reversible with time (Ruf and Patscheke, 1995; Henn et al., 1998). In contrast, although circulating degranulated platelets rapidly lose their surface P-selectin in vivo, the activation-dependent increase in platelet surface P-selectin is not reversible over time in vitro.

Choice of antibodies

Because the expression of different antigens reflects different aspects of platelet activation, it may be preferable to use a panel of monoclonal antibodies and/or reagents. Platelet-specific monoclonal antibodies are available from several commercial sources, and can be purchased directly conjugated to fluorescein, biotin, phycoerythrin, PerCP, APC, or tandem conjugates (e.g., phycoerythrin-Cy5 or RED-670). Alternatively, unlabeled antibodies can be FITC conjugated or biotinylated easily using commercially available kits. The use of antibodies that are directly conjugated with fluorochrome eliminates the requirement for the addition of secondary antibodies and/or reagents, thereby avoiding time-consuming additional incubations and washing procedures which, in unfixed samples, may result in artifactual in vitro activation of platelets. Furthermore, the use of secondary antibodies may result in increased background fluorescence and decreased sensitivity of the assay. Finally, the use of directly conjugated antibodies allows multi-color analysis with, for example, a number of differently conjugated murine antibodies.

Platelets can be detected in diluted whole blood by light scatter alone using flow cytometry; however, some of the particles with characteristic light scatter for platelets may not be bona fide platelet events. It is therefore recommended that a minimum two-color/two-antibody technique be used for whole-blood platelet flow cytometry, with one monoclonal antibody (e.g., anti-CD41, anti-CD61, anti-CD42a, anti-CD42b) to identify a particle as a platelet, and a second (or more) monoclonal antibody or reagent conjugated with alternative fluorochrome (or fluorochromes) to quantitate the expression of the glycoprotein of interest. Laboratories need to confirm that “home-brew” antibody cocktails of multiple antibody specificities do not interfere with the labeling of each individual monoclonal antibody.

FcyRIIa receptor-mediated activation

FcyRIIa receptors are constitutively expressed on the platelet surface. Some murine monoclonal antibodies such as those specific for CD9 and, under some conditions, a few murine monoclonal antibodies directed toward GPIIb-IIIa (CD41/CD61) can induce or augment platelet activation via FcyRIIa receptor clustering. Unintentional FcyRIIa receptor-mediated platelet activation can be avoided by using Fab murine monoclonal antibody fragments, using fix-first methods, or by blocking FcyRIIa-mediated platelet activation with the receptor-specific murine monoclonal antibody IV.3 (Medarex).

Anticipated Results

Normal healthy donors should exhibit minimal markers of platelet activation (P-selectin expression and PAC1 binding) when whole blood is handled according to the procedures described above. Antibody binding can be expressed as mean fluorescence intensity (MFI) or as the percent of platelets staining positive for a particular antibody (based on a positive analysis region placed just to the right of the negative (usually isotype) control fluorescence histogram). Depending on the experimental circumstances and the physiologic nature of the antigen being measured, either MFI or percent positive platelets may have more relevance than the other. The “percent positive platelets” method may detect subpopulations of platelets arising from a local in vivo insult; however, it is important to recognize that “antibody-positive” platelets may have very little antigen expressed on their surface. For example, in a given clinical setting, the data may be reported as 20% circulating activated platelets, based on P-selectin positivity, however, if each P-selectin-positive platelet expresses only 10% of maximal platelet surface P-selectin, then the overall increase in percent P-selectin-positive platelets may be only 2%. If the goal is to determine the relative increase of a platelet surface antigen, MFI is the preferred method of data presentation.

Submaximal doses of platelet agonists are often useful to detect subtle differences in platelet reactivity between different donor groups. Maximal doses of platelet agonist are used to determine maximal potential for platelet activation and receptor expression. Maximally expressed markers of activation serve as a benchmark for comparing platelet activation between different donors and data sets. For example, P-selectin expression may be reported as 100%
maximal expression rather than as the raw MFI value derived from the flow cytometer. Less than maximal expression may then be reported as a fraction, or percentage of maximal expression.

Controls

For activation-dependent antibodies, inclusion of a positive control sample (e.g., maximally activated by thrombin, TRAP, or phorbol myristate acetate) assists in the evaluation of surface antigen per platelet and in the comparison of platelet responsiveness between different donors. Positive control samples also ensure that platelet agonists and activation-dependent monoclonal antibodies and/or reagents are functioning properly.

Negative controls include the “blank” or isotype control commonly used in many flow cytometric immunophenotyping assays, as well as the “no-agonist” control. The isotype control defines the fluorescent events that are considered negative versus those considered positive. This negative control takes into account cellular autofluorescence, Fc-mediated antibody binding, and nonspecific antibody binding. Defining the negative and positive regions may be determined by a fluorochrome-matched, concentration-matched, F/P ratio-matched, and isotype-matched antibody to an irrelevant receptor. To determine the negative versus positive regions for PAC1 binding, an appropriate negative control may include an RGD-based peptide in sufficient concentration to completely block specific PAC1 binding. For annexin V, factor V/Va, and/or factor X/Xa binding, omitting the addition of Ca²⁺ may be used to define negative and positive regions. The “no-agonist” control provides information about technique as well as about donor or patient in vivo platelet activation status, since improper sample handling will lead to artificial platelet activation. Other controls might include establishing normal ranges for activation markers.

Time Considerations

To minimize delays, prepare working concentrations of monoclonal antibody reagents, platelet agonists, buffers, and fixatives just prior to receiving whole blood for platelet testing. Reagent preparation should take ~15 min. Platelet activation and labeling with monoclonal antibodies should proceed for ~20 min and subsequent fixation for ~15 min. Note that for the determination of procoagulant platelets (see Basic Protocol 3) there are two incubation steps of 20 min each. Each sample can be analyzed in usually <1 min; therefore, the total time for whole-blood flow-cytometric platelet analysis is generally ~1 hr.

Literature Cited


Contributed by Lori A. Krueger, Marc R. Barnard, A.L. Frelinger III, Mark I. Furman, and Alan D. Michelson University of Massachusetts Medical School Worcester, Massachusetts
11-dehydro Thromboxane B₂ EIA Kit

Catalog No. 519501

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If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

PRECAUTIONS

**WARNING:** This product is not intended or approved for use in humans or veterinary animals. Reliance on this product for analyte measurements in a therapeutic setting is hazardous and may result in illness or injury.

- Please read these instructions carefully before beginning this assay.
- The reagents in this kit have been tested and formulated to work exclusively with ACE™ EIA kits. This kit may not perform as described if any reagent or procedure is replaced or modified.
- For research use only. Not for human or diagnostic use.

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Cayman Chemical Company makes no warranty of any kind, expressed or implied, including, but not limited to, the warranties of fitness for a particular purpose and merchantability, which extends beyond the description of the chemicals on the face hereof, except that the material will meet our specifications at the time of delivery. Buyer’s exclusive remedy and Cayman Chemical Company’s sole liability hereunder shall be limited to refund of the purchase price of, or at Cayman Chemical Company’s option, the replacement of, all material that does not meet our specifications. Cayman Chemical Company shall not be liable otherwise or for incidental or consequential damages, including, but not limited to, the costs of handling. Said refund or replacement is conditioned upon Buyer giving written notice to Cayman Chemical Company within thirty (30) days after arrival of the material at its destination. Failure of Buyer to give said notice within said thirty (30) days shall constitute a waiver by Buyer of all claims hereunder with respect to said material.

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Cayman Chemical offers an introductory course in EIA theory and practice. Please contact our Customer Service Department for more information.
STORAGE AND STABILITY
This kit will perform as specified if stored at -20°C and used before the expiration date indicated on the outside of the box.

ADDITIONAL ITEMS REQUIRED
1. A plate reader with a 405-420 nm filter.
2. An adjustable pipetor.
3. A source of “UltraPure” water. Water used to prepare all EIA reagents and buffers must be deionized and free of trace organic contaminants (“UltraPure”). Use activated carbon filter cartridges or other organic scavengers. Glass distilled water (even if double distilled), HPLC-grade water, and sterile water (for injections) are not adequate for EIA. [NOTE: UltraPure water is available for purchase (Catalog No. 400000).]
4. Materials used for sample preparation (see page 6).

ABOUT THIS ASSAY
Thromboxane A2 (TXA2) is produced from arachidonic acid by many cells and causes irreversible platelet aggregation and vascular and bronchial smooth muscle contraction.1-3 TXA2 is rapidly hydrolyzed non-enzymatically to form TXB2. Although it is common to estimate TXA2 levels by measuring TXB2, most of the TXB2 measured in plasma or urine is due to ex vivo platelet activation or intra-renal production, respectively.4 Measurement errors are compounded by the fact that normal concentrations of circulating TXB2 are extremely low (1-2 pg/ml), and highly transient (t1/2 = 5-7 minutes).5,6 To circumvent this problem, it is necessary to measure a metabolite that cannot be formed by platelets or by the kidney. TXB2 can be metabolized by 11-hydroxy thromboxane dehydrogenase to form 11-dehydro TXB2, or by β-oxidation to form 2,3-dinor TXB2.7 Infusion studies using TXB2 have shown that both metabolites are formed equally, although 11-dehydro TXB2 has a longer circulating half-life (t1/2 = 45 minutes).6,8 Therefore, measurement of 11-dehydro TXB2 in plasma or urine will give a time-integrated indication of TXA2 production.

Normal plasma levels of 11-dehydro TXB2 (1-2 pg/ml) are below the detection limit of this assay (10 pg/ml), so plasma samples must be purified and concentrated prior to analysis.9 Because urine contains many possible crossreacting eicosanoid metabolites, it is recommended to purify urine also (see purification protocol, page 6). This assay has been validated by comparison to GC/MS.10,11

INTRODUCTION TO ACE™ EIAs
Description of the ACE™ Competitive Enzyme Immunoassay12,13
This assay is based on the competition between 11-dehydro TXB2 and a 11-dehydro TXB2-acetylcholinesterase (AChE) conjugate (11-dehydro TXB2 tracer) for a limited number of 11-dehydro TXB2-specific rabbit antiserum binding sites. Because the concentration of the 11-dehydro TXB2 tracer is held constant while the concentration of 11-dehydro TXB2 varies, the amount of 11-dehydro TXB2 tracer that is able to bind to the rabbit antiserum will be inversely proportional to the concentration of 11-dehydro TXB2 in the well. This rabbit antiserum-11-dehydro TXB2 (either free or tracer) complex binds to the mouse monoclonal anti-rabbit IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents, and then Ellman’s Reagent (which contains the substrate to AChE) is added to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 412 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of 11-dehydro TXB2 tracer bound to the well, which is inversely proportional to the amount of free 11-dehydro TXB2 present in the well during the incubation; or

\[
\text{Absorbance} = \frac{\text{Bound 11-dehydro TXB2 Tracer}}{11\text{-dehydro TXB2}}
\]

A schematic of this process is shown in Figure 1 (see page 4).
Biochemistry of ACE\textsuperscript{TM} EIAs

The electric organ of the electric eel, \textit{Electrophorus electricus}, contains an avid AChE capable of massive catalytic turnover during the generation of its electrochemical discharges. The electric eel AChE has a clover leaf-shaped tertiary structure consisting of a triad of tetramers attached to a collagen-like structural fibril. This stable enzyme is capable of high turnover (64,000 s\textsuperscript{-1}) for the hydrolysis of acetylthiocholine.

A molecule of the analyte covalently attached to a molecule of AChE serves as the tracer in ACE\textsuperscript{TM} enzyme immunoassays. Quantification of the tracer is achieved by measuring its AChE activity with Ellman’s Reagent. This reagent consists of acetylthiocholine and 5,5'-dithiobis-(2-nitrobenzoic acid). Hydrolysis of acetylthiocholine by AChE produces thiocholine (see Figure 2, page 5). The non-enzymatic reaction of thiocholine with 5,5'-dithiobis-(2-nitrobenzoic acid) produces 5-thio-2-nitrobenzoic acid, which has a strong absorbance at 412 nm ($\epsilon = 13,600$).

AChE has several advantages over other enzymes commonly used for enzyme immunoassays. Unlike horseradish peroxidase, AChE does not self-inactivate during turnover. This property of AChE also allows multiple development of the assay if it is accidentally splashed or spilled. In addition, the enzyme is highly stable under the assay conditions, has a wide pH range (pH 5-10), and is not inhibited by common buffer salts and preservatives. Since AChE is stable during the development step, it is unnecessary to use a “stop” reagent, and the plate may be read whenever it is convenient.
Definition of Key Terms

Blank: background absorbance caused by Ellman’s Reagent. Even freshly prepared Ellman’s Reagent has some measurable absorbance, approximately 0.1 Absorbance Units (A.U.). The blank absorbance should be subtracted from the absorbance readings of all the other wells.

Total Activity: total enzymatic activity of the AChE-linked tracer. This is analogous to the specific activity of a radioactive tracer.

NSB (Non-Specific Binding): non-immunological binding of the tracer to the well. Even in the absence of specific antiserum a very small amount of tracer still binds to the well: the NSB is a measure of this low binding.

\( B_0 \) (Maximum Binding): maximum amount of the tracer that the antiserum can bind in the absence of free analyte.

\( \%B/B_0 \) (%Bound/Maximum Bound): ratio of the absorbance of a particular sample or standard well to that of the maximum binding (\( B_0 \)) well.

Standard Curve: a plot of the \( \%B/B_0 \) values versus concentration of a series of wells containing various known amounts of analyte.

**PRE-ASSAY PREPARATION**

Water used to prepare all EIA reagents and buffers must be deionized and free of trace organic contaminants ("UltraPure"). Use activated carbon filter cartridges or other organic scavengers. Glass distilled water (even if double distilled), HPLC-grade water, and sterile water (for injections) are not adequate for EIA. UltraPure water may be purchased from Cayman Chemical (Catalog No. 400000).

Buffer Preparation (Store all buffers at 4°C)

1. 11-dehydro Thromboxane \( B_2 \) EIA Buffer Preparation

Dilute the contents of one vial of EIA Buffer Concentrate (vial #4) with 90 ml of UltraPure water. Be certain to rinse the vial to remove any salts that may have precipitated. **(NOTE: It is normal for the concentrated buffer to contain crystalline salts after storing. These will completely dissolve upon dilution with water.)**
2. **Wash Buffer Preparation**

Dilute the contents of the vial (5 ml) of Wash Buffer Concentrate (vial #5) to a total volume of 2 liters with UltraPure water and add 1 ml of Tween 20 (vial #5a), or dilute the contents of the vial (12.5 ml) of Wash Buffer Concentrate (vial #5) to a total volume of 5 liters with UltraPure water and add 2.5 ml of Tween 20 (vial #5a). **(NOTE: Tween 20 is a viscous liquid and cannot be measured by a pipette. A positive displacement device such as a syringe should be used to deliver small quantities accurately.)** A smaller volume of Wash Buffer can be prepared by diluting the Wash Buffer Concentrate 1:400 and adding Tween 20 (0.5 ml/liter of Wash Buffer).

**Sample Preparation**

Proper sample storage and preparation are essential for consistent and accurate results. Please read this section thoroughly before beginning the assay. **(NOTE: 11-dehydro TXB2 is capable of existing in two different conformations. The 11-dehydro TXB2 ELA Buffer supplied with this kit is designed to convert all of the 11-dehydro TXB2 into one conformation for more consistent results. All standards and samples should be diluted using this buffer (unpurified samples at least 1:2), and kept at room temperature for two hours before aliquotting to the plate.)**

**General Precautions**

1. All samples must be free of organic solvents prior to assay.
2. Samples should be assayed immediately after collection; samples that cannot be assayed immediately should be treated as described below and stored at -80°C.

1. **Urine Samples**

Dilutions of 1:2 and greater show a direct linear correlation between 11-dehydro TXB2 immunoreactivity and 11-dehydro TXB2 concentration. However, there are many eicosanoid metabolites in urine that may crossreact with this assay. To ensure accurate results, urine samples should be purified prior to assay (see protocol below). Incubate urine samples briefly on ice and centrifuge to remove precipitate prior to purification.

2. **Plasma Samples**

Plasma is a complex matrix that contains many substances that can interfere with this assay. We have found that plasma samples must be diluted at least 1:50 in 11-dehydro TXB2 ELA Buffer before interference is minimized and consistent results are obtained. However, at this dilution the 11-dehydro TXB2 content of normal samples (1-2 pg/ml) will be well below the detection limit of this assay. These samples must be purified prior to assay (see the purification protocol below). By subjecting a large volume of sample (5-10 ml) to this procedure, the 11-dehydro TXB2 content can be concentrated into as little as 0.5 ml of 11-dehydro TXB2 ELA Buffer. This will bring the 11-dehydro TXB2 concentration into the readable range of the standard curve.

Plasma samples should be collected in vacutainers containing sodium heparin, EDTA, or sodium citrate. Vacutainers can also be supplemented with indomethacin to give a final concentration of at least 10 μM. Indomethacin will prevent ex vivo formation of eicosanoids, which have the potential to interfere with this assay (although most eicosanoids do not appear to exhibit any cross-reactivity (see page 10)).

**Purification Protocol**

Urine, plasma, serum, and whole blood, as well as other heterogeneous mixtures, such as lavage fluids and aspirates, often contain contaminants which can interfere in the assay. It is best to check for interference before embarking on a large number of sample measurements.

To test for interference, dilute one or two test samples to obtain at least two different dilutions of each sample between approximately 10 and 200 pg/ml (i.e., between 20-80 B/B0). If the two different dilutions of the sample show good correlation (differ by 20% or less) in the final calculated 11-dehydro-TXB2 concentration, purification is not required. If you do not see good correlation of the different dilutions, purification is advised. The procedure below is a simple solid phase extraction method designed specifically for purification 11-dehydro-TXB2. This method has been validated in urine samples.

**Additional solutions needed:**

1. Buffer A: 63 mM ammonium bicarbonate, pH 8.6
2. Buffer B: 0.63 mM ammonium bicarbonate, pH 8.6
3. Elution Solution: Methanol containing 2% formic acid (made fresh just before use)
Procedure (based on 1 ml of sample)
1. Dilute centrifuged samples with an equal volume of Buffer A and incubate at room temperature for 3 hours.
2. Condition a SPE cartridge (mixed bed) (Catalog No. 400021) with 3 ml methanol, followed by 3 ml deionized water. Do not allow the column to dry.
3. Apply the sample to the column using gravity flow.
4. Rinse the column sequentially with 3 ml of Buffer B, 3 ml deionized water, and then with 6 ml methanol.
5. Elute the 11-dehydro-TXB₂ using 2 ml of elution solution [NOTE: It is critical that the elution solution be used within 1-2 hours of preparation.]
6. Evaporate the sample to dryness under a gentle stream of dry nitrogen.
7. Resuspend the sample in 1 ml of 11-dehydro-TXB₂ EIA buffer and incubate at room temperature for 3 hours prior to use in the EIA.

Preparation of Assay-specific Reagents
1. 11-dehydro Thromboxane B₂ Standard
   Equilibrate a pipet tip in ethanol by repeatedly filling and expelling the tip with ethanol several times. Using the equilibrated pipet tip, transfer 100 μl of the 11-dehydro TXB₂ Standard (vial #3) into a clean test tube, then dilute with 900 μl UltraPure water. The concentration of this solution (the bulk standard) will be 5 ng/ml.
   To prepare the standard for use in EIA: Obtain 8 clean test tubes and number them #1 through #8. Aliquot 900 μl 11-dehydro TXB₂ EIA Buffer to tube #1 and 500 μl 11-dehydro TXB₂ EIA Buffer to tube #2-8. Transfer 100 μl of the bulk standard (5 ng/ml) to tube #1 and mix thoroughly. Serially dilute the standard by removing 500 μl from tube #1 and placing in tube #2; mix thoroughly. Next, remove 500 μl from tube #2 and place it into tube #3; mix thoroughly. Repeat this process for tubes #4-8. Incubate these standards at room temperature for a minimum of 2 hours prior to aliquoting to the plate. These diluted standards should not be stored for more than 24 hours.
2. 11-dehydro Thromboxane B₂ AChE Tracer
   Reconstitute the 100 dtm 11-dehydro TXB₂ Tracer (vial #2) with 6 ml 11-dehydro TXB₂ EIA Buffer or the 500 dtm 11-dehydro TXB₂ Tracer (vial #2) with 30 ml 11-dehydro TXB₂ EIA Buffer. Store the reconstituted 11-dehydro TXB₂ Tracer at 4°C (do not freeze) and use within four weeks. A 20% surplus of 11-dehydro TXB₂ Tracer has been included to account for any incidental losses.
   Tracer Dye Instructions (Optional)
   This dye may be added to the tracer, if desired, to aid in visualization of tracer-containing wells. Add the dye to the reconstituted tracer at a final dilution of 1:100 (add 60 μl of dye to 6 ml tracer or add 300 μl of dye to 30 ml of tracer).
3. 11-dehydro Thromboxane B₂ Antiserum
   Reconstitute the 100 dtm 11-dehydro TXB₂ Antiserum (vial #1) with 6 ml 11-dehydro TXB₂ EIA Buffer or the 500 dtm 11-dehydro TXB₂ Antiserum (vial #1) with 30 ml 11-dehydro TXB₂ EIA Buffer. Store the reconstituted 11-dehydro TXB₂ Antiserum at 4°C. It will be stable for at least four weeks. A 20% surplus of 11-dehydro TXB₂ Antiserum has been included to account for any incidental losses.
   Antiserum Dye Instructions (Optional)
   This dye may be added to the antiserum, if desired, to aid in visualization of antiserum-containing wells. Add the dye to the reconstituted antiserum at a final dilution of 1:100 (add 60 μl of dye to 6 ml antiserum or add 300 μl of dye to 30 ml of antiserum).
PERFORMING THE ASSAY

Plate Set Up

The 96 well plate(s), included with this kit, is supplied ready to use. It is not necessary to rinse the plate(s) prior to adding the reagents. [NOTE: If you do not need to use all the strips at once, place the unused strips back in the plate packet and store at 2-8°C. Be sure the packet is sealed with the desiccant inside.]

Each plate or set of strips must contain a minimum of two blanks (Blk), two non-specific binding wells (NSB), two maximum binding wells (Bm), and an eight point standard curve run in duplicate. [NOTE: Each assay must contain this minimum configuration in order to ensure accurate and reproducible results.] At a minimum, each sample should be assayed at two dilutions and each dilution should be assayed in duplicate. For statistical purposes, we recommend assaying samples in triplicate.

A suggested plate format is shown in Figure 3, below. The user may vary the location and type of wells present as necessary for each particular experiment. The plate format provided below has been designed to allow for easy data analysis using a convenient spreadsheet offered by Cayman Chemical (see Calculating the Results on page 9 for more details). We suggest you record the contents of each well on the template sheet provided (see page 12).

Full Plate Format

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</tbody>
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Blk - Blank
TA - Total Activity
NSB - Non-Specific Binding
Bm - Maximum Binding
S1-S8 - Standards 1-8
1-24 - Samples

![Figure 3. Sample plate format](image)

Pipet the Reagents

**PIPETTING HINTS**

- Use different tips to pipet the buffer, standard, sample, tracer, and antibody.
- Before pipetting each reagent, equilibrate the pipet tip in that reagent (i.e., slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipet tip to the reagent(s) already in the well.

1. 11-dehydro Thromboxane B₂ EIA Buffer
   - Add 100 µl 11-dehydro TXB₂ EIA Buffer to Non-Specific Binding (NSB) wells. Add 50 µl 11-dehydro TXB₂ EIA Buffer to Maximum Binding (Bm) wells.

2. 11-dehydro Thromboxane B₂ Standard
   - After the standards have been diluted and incubated in 11-dehydro TXB₂ EIA Buffer for at least 2 hours, they may be aliquoted to the plate. Add 50 µl from tube #8 to both of the lowest standard wells (S8). Add 50 µl from tube #7 to each of the next two standard wells (S7). Continue with this procedure until all the standards are aliquoted. The same pipet tip should be used to aliquot all the standards. Before pipetting each standard, be sure to equilibrate the pipet tip in that standard.

3. Samples
   - Add 50 µl of sample per well. Each sample should be assayed at a minimum of two dilutions. Each dilution should be assayed in duplicate (triplicate recommended).
4. 11-dehydro Thromboxane B₂ AChE Tracer
   Add 50 µl to each well except the Total Activity (TA) and the Blank (Blk) wells.
5. 11-dehydro Thromboxane B₂ Antiserum
   Add 50 µl to each well except the Total Activity (TA), the Non-Specific Binding (NSB), and the Blank (Blk) wells.

<table>
<thead>
<tr>
<th>Well</th>
<th>EIA Buffer</th>
<th>Standard/Sample</th>
<th>Tracer</th>
<th>Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blk</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TA</td>
<td>-</td>
<td>-</td>
<td>5 µl (x dev. step)</td>
<td>-</td>
</tr>
<tr>
<td>NSB</td>
<td>100 µl</td>
<td>-</td>
<td>50 µl</td>
<td>-</td>
</tr>
<tr>
<td>B₀</td>
<td>50 µl</td>
<td>-</td>
<td>50 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>Sd/Sample</td>
<td>-</td>
<td>50 µl</td>
<td>50 µl</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

*Table 1. Quick Reference Pipetting Summary*

Incubate the Plate
Cover each plate with plastic film (item #7) and incubate for 18 hours at room temperature.

Develop the Plate
When ready to develop the plate(s), reconstitute one 100 dtl vial of Ellman's Reagent (vial #8) with 20 ml of UltraPure water, or reconstitute one 250 dtl vial of Ellman's Reagent (vial #8) with 50 ml of UltraPure water (20 ml of reagent is sufficient to develop 100 wells). Reconstituted Ellman's Reagent is unstable and should be used the same day it is prepared; protect the Ellman's Reagent from light when not in use. Extra vials of the reagent have been provided if a plate needs to be re-developed or multiple assays need to be run on different days.

Empty the wells and rinse five times with Wash Buffer. Add 200 µl of Ellman's Reagent to each well and 5 µl of tracer to the Total Activity wells. Cover the plate with plastic film.

Optimum development is obtained by using an orbital shaker equipped with a large, flat cover to allow the plate(s) to develop in the dark. This assay typically develops (i.e., B₀ wells equal 0.3 A.U. (blank subtracted)) in 60-90 minutes.

Read the Plate
Read the plate(s) at a wavelength between 405 and 420 nm (usually 412 nm). Before reading each plate, wipe the bottom of the plate with a clean tissue to remove finger prints, dirt, etc., as smudges on the bottom of the plate can significantly alter absorbance readings. Be certain that the Ellman's Reagent has not splashed up on the plate cover as any loss of Ellman's Reagent will affect the absorbance readings. If it did, use a pipet to remove the Ellman's Reagent from the cover and place into the well. If too much Ellman's Reagent has splashed on the cover to easily redistribute back into the wells, wash the plate 3 times with Wash Buffer and repeat the development with fresh Ellman's Reagent.

The plate may be checked periodically until the B₀ wells have reached a minimum of 0.3 A.U. (blank subtracted). The plate should be read when the absorbance of the B₀ wells is in the range of 0.3-0.8 A.U. (blank subtracted). If the absorbance of the wells exceeds 1.5, wash the plate, add fresh Ellman's Reagent, and let it develop again.

**CALCULATING THE RESULTS**

It is usually more convenient to calculate the assay results by computer; most plate readers come with data reduction software, or a spreadsheet program can be used (4-parameter logistic or log-log curve fit). [Cayman Chemical has a computer spreadsheet available for data analysis. Please contact Technical Service or visit our website (www.caymanchem.com/ia LOSS/promo/kits) for more information or to obtain a free copy of this convenient data analysis tool.] If the results need to be calculated manually, the procedure is as follows:

**[NOTE: If the plate reader has not subtracted the absorbance readings of the blank wells from the absorbance readings of the rest of the plate, be sure to do that now.]**

1. Average the absorbance readings from the NSB wells.
2. Average the absorbance readings from the B₀ wells.
3. Subtract the NSB average from the B₀ average. This is the corrected B₀ or corrected maximum binding.
4. Calculate the \%B/B₀ (\% Sample or Standard Bound/Maximum Bound) for the remaining wells. To do this, subtract the average NSB absorbance from the S1 absorbance and divide by the corrected B₀ (from Step 3). Multiply by 100 to obtain \%B/B₀. Repeat for S2-S8 and all sample wells.

5. The total activity values are not used in the standard curve calculations. Rather, they are used as a diagnostic tool; the corrected B₀ divided by the actual TA (10X measured absorbance) will give the % Bound. This value should closely approximate the % Bound that can be calculated from the sample data (see below). Erratic absorbance values and a low (or no) % Bound could indicate the presence of organic solvents in the buffer or other technical problems (see page 11 for Troubleshooting).

Plotting the Standard Curve
Plot \%B/B₀ for standards S1-S8 versus 11-dehydro TXB₂ concentration (usually in pg/ml) on semi-log paper.

Determining the Concentration of your Samples
Calculate the \%B/B₀ value for each sample. Determine the concentration of each sample by identifying the \%B/B₀ on the standard curve and reading the corresponding values on the x-axis. [NOTE: Remember to account for any dilutions of the sample prior to the addition to the well] \%B/B₀ values greater than 80% and less than 20% should be re-assayed as they generally fall out of the linear range of the standard curve. A 20% or greater disparity between the apparent concentration of two different dilutions of the same sample would indicate interference which could be eliminated by purification.

PERFORMANCE CHARACTERISTICS

Precision
The intra- and interassay CV's have been determined at multiple points on the standard curve; these data are summarized in the graph below.

Specificity
11-dehydro Thromboxane B₂ 100% Leukotriene B₄ <0.01%
Prostaglandin D₂ 0.99% 6-keto Prostaglandin F₁α <0.01%
Prostaglandin E₂ 0.04% Tetrnor PGE₂ <0.01%
Thromboxane B₂ 0.03% Tetrnor PGE₂ <0.01%
2,3-dinor Thromboxane B₂ 0.01%  

Sample Data
The standard curve presented here is an example of the data typically produced with this kit; however, your results will not be identical to these. You must run a new standard curve - do not use this one to determine the values of your samples. Depending on the development conditions and the purity of the water used, your results could differ substantially from the data presented below. [NOTE: Raw data is reported in milli-Absorbance Units.]

<table>
<thead>
<tr>
<th>Total Activity</th>
<th>Raw Data</th>
<th>Average</th>
<th>Corrected</th>
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<tbody>
<tr>
<td>NSB</td>
<td>6</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>B₀</td>
<td>294</td>
<td>311</td>
<td>300.5</td>
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<tr>
<td>298</td>
<td>299</td>
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<table>
<thead>
<tr>
<th>Dose (pg/ml)</th>
<th>Raw Data</th>
<th>Corrected</th>
<th>%B/B₀</th>
</tr>
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<tr>
<td>500</td>
<td>53</td>
<td>48</td>
<td>43</td>
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<td>125</td>
<td>105</td>
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<tr>
<td>62.5</td>
<td>142</td>
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<td>15.6</td>
<td>217</td>
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<tr>
<td>7.8</td>
<td>255</td>
<td>250</td>
<td>256</td>
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<tr>
<td>3.9</td>
<td>272</td>
<td>267</td>
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50% B/B₀ - 53 pg/ml
Detection Limit (80% B/B₀) - 10 pg/ml
TROUBLESHOOTING

Problem: Erratic values; dispersion of duplicates.
Causes: Trace organic contaminants in the water source; replace activated carbon filter or change source of UltraPure water. -or- Poor pipetting/technique.

Problem: High NSB (>0.035).
Causes: Poor washing. -or- Exposure of NSB wells to specific antibody.

Problem: Very low Bb.
Causes: Contamination of water with organic solvents. -or- Plate requires additional development time. -or- Dilution error in preparing reagents.

Problem: Low sensitivity (shift in dose response curve).
Causes: Standard is degraded.

Problem: Analyses of two dilutions of a biological sample do not agree (i.e., more than 20% difference).
Causes: Interfering substances are present. Sample must be purified prior to analysis by EIA.

Problem: Only Total Activity (TA) wells develop.
Causes: Trace organic contaminants in the water source; replace activated carbon filter or change source of UltraPure water.

REFERENCES


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ADDITIONAL READING

RELATED PRODUCTS
UltraPure Water - Cat. No. 400000 • 6-keto Prostaglandin F₁α EIA Kit - Cat. No. 515211 • 6-keto Prostaglandin F₁α EIA Kit (Solid Plate) - Cat. No. 515211.1 • Thromboxane B₂ EIA Kit - Cat. No. 519031 • Thromboxane B₂ EIA Kit (Solid Plate) - Cat. No. 519031.1 • 2,3-dinor Thromboxane B₂ EIA Kit - Cat. No. 519051 • 2,3-dinor Thromboxane B₂ EIA Kit (Solid Plate) - Cat. No. 519051.1 • 11-dehydro Thromboxane B₂ EIA Kit (Solid Plate) - Cat. No. 519501.1

PLATE TEMPLATE

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+---+---+---+---+---+---+---+---+
|   |  |  |  |  |  |  |  |
| A |   |   |   |   |   |   |   |
+---+---+---+---+---+---+---+---+
| B |   |   |   |   |   |   |   |
+---+---+---+---+---+---+---+---+
| C |   |   |   |   |   |   |   |
+---+---+---+---+---+---+---+---+
| D |   |   |   |   |   |   |   |
+---+---+---+---+---+---+---+---+
| E |   |   |   |   |   |   |   |
+---+---+---+---+---+---+---+---+
| F |   |   |   |   |   |   |   |
+---+---+---+---+---+---+---+---+
| G |   |   |   |   |   |   |   |
+---+---+---+---+---+---+---+---+
| H |   |   |   |   |   |   |   |
+---+---+---+---+---+---+---+---+
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NOTES

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Protocol: A Prospective Randomized Multicenter Study Comparing the Safety And Efficacy of Renal Artery Stenting With and Without the use of a Distal Protection Device (AngioGuard) and With and Without the use of a Platelet Aggregator Inhibitor (Abciximab/Reopro) (RESIST)

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Version 4.3
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<td></td>
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<td></td>
</tr>
</tbody>
</table>
Aims

The aims of this study, conducted in patients with renovascular hypertension undergoing renal artery stenting, are:

1) To determine whether embolic protection with the Angioguard XP Short Tip device during implantation of the Genesis stent results in:
   • Retrieval of atheroembolic material
   • Improved renal function 1 month after the procedure
   • Decreases evidence of inflammation or injury in the kidney(s)
   • Is safe

2) To determine whether inhibition of the glycoprotein IIbIIIa receptor with Abciximab results in:
   • Improved renal function 1 month after the procedure
   • Decreases evidence of inflammation or injury in the kidney(s)
   • Is safe

Introduction

Renal artery stenosis may account for up to 5% of all cases of hypertension (1-3), and is an important cause of renal failure (4-6). The diagnosis of renal artery stenosis is growing more common as the population ages (7). Prior studies have demonstrated that successful surgical revascularization results in improved blood pressure control but is limited by adverse cardiovascular events in the perioperative period (1,8-10). Renal artery angioplasty with endovascular stent placement has become the dominant mode of therapy.

A limitation of renal stenting is the occurrence of renal insufficiency after the procedure. Several etiologies have been implicated in this process including atheroembolization or platelet embolization to the kidney, and contrast nephropathy. Recently, an endovascular filter has been developed, the Angioguard XP Short Tip device. This device may be able to limit post-procedural renal dysfunction by 1) preventing atheroembolization, and 2) diminishing renal contrast exposure by limiting blood flow into the affected kidney.
During coronary stenting, inhibitors of the platelet glycoprotein IIb/IIa receptor reduce the incidence of myocardial necrosis, presumably due to inhibition of platelet aggregation and subsequent platelet emboli. One of these agents, Abciximab, has demonstrated efficacy in several trials of coronary intervention. Whether these agents confer a similar benefit during renal stenting remains untested and unknown. In animal models activation of platelets contribute to glomerular injury (90) whereas inhibition of platelet reduces such injury (91). Importantly, Abciximab may be the ideal agent for this application since the other approved gp IIb/IIIa inhibitors, integrilin and tirofiban, have limited utility or are contraindicated in patients with renal dysfunction. Reopro™ (abciximab) is a human/murine genetic reconstruction derived from the murine monoclonal 7E3 antibody that binds selectively to the glycoprotein IIb/IIIa platelet receptor. The molecule contains v.3 murine heavy and light chain variable regions, which confer antibody specificity, linked to human heavy and light chain constant domains. Abciximab is supplied as a sterile, nonpyrogenic solution containing 2 mg of abciximab (monoclonal c7E3 Fab) per mL of 0.15 M sodium chloride, 0.01 M sodium phosphate, and 0.001% Polysorbate 80 at a pH of 7.2. The product has been screened for the presence of murine viruses. The solution contains no preservatives. The placebo solution is 0.15 M sodium chloride, 0.01 M sodium phosphate and 0.001% Polysorbate 80 at a pH of 7.2.

Renal artery stenting

In 1974, Hunt et al retrospectively compared two similar but nonrandomized groups of 100 patients each treated either medically or surgically for renovascular hypertension. The surgical group had a significantly lower rate of mortality, stroke, heart attack, and azotemia, and had better blood pressure control than the medical group (49). Chaikof et al reviewed the results of surgical revascularization over ten years (50). They reported an improvement in GFR in 42% of patients, worsening in 4% and no change in 54%. High blood pressure was either cured or brought under better control in 50% of patients. Since these studies predated the advent of more efficacious anti-hypertensive medications, the applicability to present day management remains limited.
Surgery has also been associated with significant perioperative complications including mortality. Most patients with renal artery stenosis have lesions in other vascular beds making them higher risk operative candidates. Thus, percutaneous renal angioplasty (PTRA) is an attractive alternative. PTRA has been associated with improved blood pressure control and a decrease in need for antihypertensive medications (51) in retrospective studies. However, PTRA has a high restenosis rate for ostial lesions, and ostial lesions comprise 80-85% of all atherosclerotic renal artery lesions (52-55).

With the advent of endovascular stents, these problems can be circumvented. Rees et al reported 96% technical success rate with Palmaz stents in ostial lesions (55). In recent years, there have been other prospective cohort studies which report good success rates for PTRA with stent deployment (54,56). In summary, the results of revascularization with stenting appear to be comparable to that of surgery with less immediate morbidity and mortality.

Assessment of Renal Function

The effect of either distal atheroembolic protection or antiplatelet therapy on renal function, specifically glomerular filtration rate (GFR), has not been evaluated. Thus, it is not clear whether these strategies preserve renal function. While markers of global renal function such as serum blood urea nitrogen and creatinine are helpful in estimating GFR, neither are specific for the affected kidney. Thus they are unhelpful in quantifying the exact effect of treatment on GFR in the affected kidney.

Furthermore, both blood urea nitrogen and creatinine exhibit some degree of tubular reabsorption, and are therefore inaccurate markers of renal clearance. The glomerular filtration rate (GFR) will be measured by non-radioactive iohexol clearance. Iohexol concentrations will be measured by high performance liquid chromatography (HPLC) in serum samples obtained at specific intervals during the baseline and one month evaluations. (the specific time for the serum samples will be determined by a locally estimated GRF with a serum creatinine) following intravenous injection of iohexol. The addition of dynamic renal scintography (DRS) with depth estimates using 99mTc-diethylenetriamine pentaacetic acid (DTPA) can
quantify the specific contribution of each kidney to total GFR. The combination of these two methods may provide a superior assessment of renal function of the affected kidney.

Additional information about the health of the kidney can be learned from urinalysis and analysis of sera. Specifically, injury to the kidney can be identified in this manner. Markers for inflammation, injury, fibrosis, and platelet activation such as lactate dehydrogenase release (92-94) and thromboxane A2 (91) can be assayed for.

Preliminary Data

Renal insufficiency after renal stenting may have profound implications on late patient survival. Recent unpublished data from our lab suggests an important relationship between the development of renal insufficiency after renal artery stenting with late mortality (Figure 1). In patients starting with normal renal function, the development of renal insufficiency was associated with an increase in mortality from 8 to 18% over a 19±20 month follow-up period. Whether this is related to atheroembolization, platelet aggregation or contrast-induced renal insufficiency remains unknown.
Figure 1: Kaplan-Meier Cumulative Survival Plot for Patients With Renovascular Disease

Kaplan-Meier cumulative survival plot for patients with renovascular disease grouped by assessment of renal function at baseline and follow-up. Survival is ranked from best to worst as follows: NL-NL, ABNL-NL, ABNL-ABNL, NL-ABNL.

Over 19±20 mo, 40 known deaths occurred (17%). The following differences were more frequent in patients that died during follow-up when compared to survivors: increased baseline creatinine (CR) (2.1±1.2 mg/dL versus 1.4±1.1 mg/dL, p<0.05), increased follow-up CR (2.4±1.6 mg/dL versus 1.5±1.0 mg/dL, p<0.0001), presence of bilateral disease or a solitary kidney (BD/SK) (55% versus 36%, p<0.05), decreased follow-up systolic BP (137±27 mmHg versus 149±27 mmHg, p<0.05), history of CHF (68% versus 29%, p<0.0001), and history of previous MI (53% versus 29%, p<0.05). A comparison for baseline and follow-up characteristics of deceased and living patients is shown in Tables I and II.
**Table I.** Baseline Characteristics of the Patients based on survival. Values for the entire cohort are provided for reference only, and are not included in the unpaired t-test and Chi-square analyses.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Entire Cohort (n=232)</th>
<th>Deceased Patients (n=40)</th>
<th>Alive Patients (n=155)</th>
<th>p-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex – no. (%)</td>
<td>97 (42%)</td>
<td>21 (53%)</td>
<td>59 (38%)</td>
<td>.0980</td>
</tr>
<tr>
<td>Age – yr</td>
<td>69±11</td>
<td>74±8</td>
<td>68±11</td>
<td>.0018</td>
</tr>
<tr>
<td>White, non Hispanic (%)</td>
<td>213 (92%)</td>
<td>40 (100%)</td>
<td>140 (90%)</td>
<td>.0406</td>
</tr>
<tr>
<td>Blood Pressure – mm Hg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>170±28</td>
<td>165±32</td>
<td>172±27</td>
<td>.1579</td>
</tr>
<tr>
<td>Diastolic</td>
<td>83±14</td>
<td>81±14</td>
<td>85±15</td>
<td>.1106</td>
</tr>
<tr>
<td>Pulse pressure</td>
<td>87±23</td>
<td>85±24</td>
<td>87±24</td>
<td>.4864</td>
</tr>
<tr>
<td>Antihypertensive drugs</td>
<td>2.3±1.0</td>
<td>2.6±1.2</td>
<td>2.2±1.0</td>
<td>.0394</td>
</tr>
<tr>
<td>CAD – no. (%)</td>
<td>191 (82%)</td>
<td>35 (88%)</td>
<td>124 (80%)</td>
<td>.2757</td>
</tr>
<tr>
<td>CHF – no. (%)</td>
<td>82 (35%)</td>
<td>27 (68%)</td>
<td>42 (27%)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>PVD – no. (%)</td>
<td>141 (61%)</td>
<td>27 (68%)</td>
<td>94 (61%)</td>
<td>.4257</td>
</tr>
<tr>
<td>BD/SK – no. (%)¶</td>
<td>91 (39%)</td>
<td>22 (55%)</td>
<td>54 (35%)</td>
<td>.0198</td>
</tr>
</tbody>
</table>

**Indication§ for Renal Angiogram**

| Hypertension – no. (%) | 208 (90%) | 30 (75%) | 146 (94%) | .0003 |
| CHF – no. (%) | 58 (25%) | 19 (48%) | 27 (17%) | <.0001 |
| Renal dysfunction – no. (%) | 58 (25%) | 21 (53%) | 30 (19%) | <.0001 |
| Other – no. (%) | 18 (8%) | 1 (3%) | 16 (10%) | .1179 |

**Laboratory and angiographic data**

| BUN | 27±20 | 40±31 | 23±16 | <.0001 |
| Serum Creatinine | 1.5±1.1 | 2.1±1.2 | 1.4±1.1 | .0016 |
| Potassium | 4.3±0.7 | 4.7±1.4 | 4.3±0.5 | .0312 |

Version 4.3
Stenosis

<table>
<thead>
<tr>
<th></th>
<th>Pre-procedure - %</th>
<th>Post-procedure - %</th>
<th>No. vessels stented</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>71±11</td>
<td>74±10</td>
<td>70±11</td>
</tr>
<tr>
<td></td>
<td>74±10</td>
<td>-2±12</td>
<td>-5±15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-1±11</td>
<td>.0228</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.4±0.6</td>
<td>1.4±0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.4±1.0</td>
<td>.0617</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.4±1.0</td>
<td>.9596</td>
</tr>
</tbody>
</table>

* Patients who were lost to follow-up were not included in the cohort of alive patients.

† p-values for deceased vs. alive patients, statistical significance is defined as p < 0.05.

¶ BD/SK refers to patients who were revascularized with either bilateral disease (BD) or a solitary kidney (SK)

§ Indication refers to the proximate reason for the initial angiographic referral.
Table II. Late Follow-up Characteristics of the Patients based on survival. Values for the entire cohort are provided for reference only, and are not included in the unpaired t-test analyses.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Entire Cohort (n=232)</th>
<th>Deceased Patients (n=40)</th>
<th>Alive* Patients (n=192)</th>
<th>p-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood Pressure – mm Hg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>147±27</td>
<td>137±27</td>
<td>150±28</td>
<td>.0085</td>
</tr>
<tr>
<td>Diastolic</td>
<td>75±15</td>
<td>72±14</td>
<td>77±14</td>
<td>.0871</td>
</tr>
<tr>
<td>Pulse pressure</td>
<td>72±23</td>
<td>65±21</td>
<td>73±24</td>
<td>.0345</td>
</tr>
<tr>
<td>Total no. medications</td>
<td>7.8±3.3</td>
<td>9.1±3.6</td>
<td>7.7±3.2</td>
<td>.0179</td>
</tr>
<tr>
<td>Antihypertensive drugs</td>
<td>2.4±1.2</td>
<td>2.6±1.2</td>
<td>2.4±1.2</td>
<td>.3201</td>
</tr>
<tr>
<td>BUN</td>
<td>28±20</td>
<td>45±34</td>
<td>24±12</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Serum Creatinine</td>
<td>1.6±1.1</td>
<td>2.4±1.6</td>
<td>1.4±0.8</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Potassium</td>
<td>4.3±0.6</td>
<td>4.3±0.9</td>
<td>4.3±0.5</td>
<td>.9914</td>
</tr>
<tr>
<td>Length of time to last</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>followup – months</td>
<td>19±20</td>
<td>14±20</td>
<td>22±21</td>
<td>.0314</td>
</tr>
</tbody>
</table>

* Patients who were lost to follow-up were not included in the cohort of alive patients.
† p-values for deceased vs. alive patients, statistical significance is defined as p < 0.05.

A Cox Proportional Hazards analysis was performed to assess the effect of renal function on survival. Serum creatinine concentration was classified as either normal (< 1.6 mg/dL) or abnormal (≥ 1.6 mg/dL) at baseline and follow-up. This yielded four groups: Normal-Normal (132 patients, 63%), Normal-Abnormal (22 patients, 10%), Abnormal-Normal (12 patients, 6%) and Abnormal-Abnormal (45 patients, 21%). Next a survival model was built to adjust for the following characteristics which could potentially introduce confounding: age, gender, bilateral disease or solitary kidney, systolic BP, diastolic BP, pulse pressure, diabetes,
hypercholesterolemia, smoking, angina, MI, CABG, CHF, CAD, PTCA, PVD, pulmonary edema, CVD, TIA, CVA, and the use of ACE-inhibitors or Beta-adrenergic blockers (because of reported mortality benefit).

Renal dysfunction at both baseline and follow-up were highly associated with mortality (p=0.0001) despite the significant influences of CHF (p=0.0028), previous MI (p=0.0361), and age (p=0.1963). A breakdown of how these factors contributed to mortality is presented in Table III. Risk ratios were calculated as follows: Renal dysfunction (RR=1.67), CHF (RR=3.17), and previous MI (RR=2.04).
Table III. Analysis of the comorbid conditions (assessed at baseline and follow-up) which contributed significantly to mortality.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Deceased</th>
<th>Alive</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renal Function</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NL-NL</td>
<td>11 (8%)</td>
<td>121 (92%)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>(n=132)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NL-ABNL</td>
<td>4 (18%)</td>
<td>18 (82%)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>(n=82)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABNL-NL</td>
<td>3 (25%)</td>
<td>9 (75%)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>(n=12)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABNL-ABNL</td>
<td>19 (38%)</td>
<td>28 (62%)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>(n=45)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHF</td>
<td>27 (33%)</td>
<td>55 (67%)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>(n=82)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no CHF</td>
<td>13 (9%)</td>
<td>137 (91%)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>(n=150)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MI</td>
<td>21 (27%)</td>
<td>56 (73%)</td>
<td>.0044</td>
</tr>
<tr>
<td>(n=77)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no MI</td>
<td>19 (12%)</td>
<td>136 (88%)</td>
<td>.0044</td>
</tr>
<tr>
<td>(n=155)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>78±8</td>
<td>68±11</td>
<td>.0033</td>
</tr>
</tbody>
</table>

Summary
Renal artery stenosis is an important cause of hypertension. Stenting has become the most common mode of therapy. However, post-procedural renal insufficiency does occur and may add significantly to the occurrence of late mortality. A recently developed device, the Angioguard, may confer significant protection from atheroembolization. Similarly, Abciximab may offer protection against platelet aggregation and embolization. The current study seeks to identify whether either agent, or both in combination, are safe and may be beneficial.
Research Design and Methods

Patient population

Up to 112 patients will be recruited from 6 sites including the Medical College of Ohio, Toledo, OH, Massachusetts General Hospital, Boston, MA, Prairie Medical Group in Springfield IL and William Beaumont Hospital in Royal Oak MI and two not yet determined sites. Twelve subjects will be enrolled during the roll-in phase (2 from each site), with 100 enrolled in the randomized phase. The subjects enrolled in the roll-in phase will not undergo protocol-driven serologic, urologic, or nuclear medicine testing prior to, or after, enrollment. For these subjects, routine clinical care, with safety monitoring, will be utilized.

Additional sites may be recruited if enrollment does not reach a minimum of 10 patients per month. Open enrollment will occur, with randomization stratified by site and baseline Cr (see randomization).

Patients may be referred for evaluation with clinical presentations including but not limited to the following:

- Hypertension
  - Difficult to control on medical therapy
  - Early or late onset
  - Severe
- Renal insufficiency alone, or:
  - Associated with ACE inhibitor use
  - Without known etiology
  - In a patient with vascular disease
- Heart failure
  - Pulmonary edema
- Angina with poorly controlled hypertension
Patients must meet the following criteria in order to be included in the study:

**Inclusion Criteria (all must be present):**

- 1 or more renal artery stenosis
- ≥ 50% and <100% by angiography, and
- Amenable to treatment
  - Lesion length <11mm, vessel diameter <5.5 mm, and distance from aorta to first bifurcation >30 mm by visual estimate, or
  - Lesion length <15mm, vessel diameter <7.5 mm (at Angioguard), and distance from aorta to first bifurcation >35 mm by visual estimate
- Written informed consent

Any one or more of the following:

- Systemic hypertension at baseline, or a history of hypertension
- Congestive heart failure at baseline, or a history of CHF
- Renal insufficiency at baseline, or a history of renal insufficiency
- Angina, or a history of angina

**Exclusion Criteria:**

- Age <18
- Pregnancy or unknown pregnancy status in female of childbearing potential
- Unable to provide informed consent
- Vascular disease of the upper or lower extremity precluding access
- Comorbid status with life expectancy ≤ 6 months
- Chronic renal failure on dialysis
- Previous kidney transplant
- Renal artery stenosis not amenable to stent placement
- Allergy to stainless steel
- Allergy to all of the following: aspirin, clopidogrel, ticlopidine
- Coexisting renal disease known to be due to: scleroderma, diabetic nephropathy, sickle cell anemia
- Participation in another drug or device trial during the study period
- Known untreated aneurysm of the abdominal aorta
- Known kidney size less than 8 cm supplied by target vessel

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- Allergy to Abciximab or heparin
- Previous stent in the artery to be treated
- Any renal stenosis not amenable to treatment with Genesis stent and Angioguard use
  - Lesion length ≥15mm (visual estimate)
  - Vessel diameter <3.5 or >8.0 mm at stent site (visual estimate)
  - Vessel diameter <3.0 or >7.5 mm at Angioguard site (visual estimate)
  - (See inclusion criteria for “amenable to treatment”)
- Active internal bleeding
- Gastrointestinal or genitourinary bleeding within 6 weeks of clinical significance
- History of stroke within 2 years or stroke with a significant residual neurologic deficit
- Bleeding diathesis
- Administration of oral anticoagulants within 7 days unless prothrombin time with INR ≤ 1.2 times control
- Thrombocytopenia: <100,000 platelets / microliter
- Major surgery or trauma within 6 weeks
- Intracranial neoplasm, arteriovenous malformation; or aneurysm
- Presumed or documented history of vasculitis
- Use of intravenous dextran before, or intended use during, intervention
- Any planned surgical or interventional procedure within the 30 day period after the index procedure
- Allergy to iodine based contrast media

Patient Management
Informed consent

Those patients eligible for enrollment will sign a written informed consent for a study protocol approved by the Institutional Review Board prior to any study-related procedures. A copy of the written informed consent document should be given to the patient and a second copy placed with the patient’s medical record. In
the source documentation, a note should be placed stating that the following elements of informed consent were adhered to:

- The patient understood the nature, purpose, and duration of their participation.
- The benefits, alternatives and risks were explained.
- The patient had questions answered and understands whom to contact for further questions or in the event of injury.
- That participation was voluntary.

However, all elements of informed consent, dictated by Federal Regulation, ICH GCP guidelines and institutional policy must be adhered to.

**Baseline evaluation**

Patients who meet inclusion and exclusion criteria will be asked to participate. All medications administered within the week prior to the procedure will be recorded on the case report form. Renal function will be assessed by the Iohexol clearance and the DTPA scan. (See specific study for preparation) Subsequently, they will have their baseline office seated blood pressure determined. Baseline blood (complete blood count, blood glucose, blood urea nitrogen, creatinine, lipid profile, lactate dehydrogenase, prothrombin time with INR (international normalized ratio) and activated partial thromboplastin time if they have not been done in the past month) and a serum sample for core lab analysis will be collected at the time of patient arrival. The patient will be asked to hold their urine for a 2 hour urine collection. This specimen will be collected and utilized for immediate urine dipstick analysis at the study site and preparation for a sample to be frozen and shipped to the core lab for analysis and a urine protein electrophoresis.

**Pre-procedural care**

Prior to the procedure, treatment with acetylcysteine may be initiated, upon the recommendation of the patient’s treating physician. The patient will be randomly assigned to use of the Angioguard device and use of Abciximab. Thus, there will be 4 study groups: Angioguard and Abciximab, Angioguard and no Abciximab, no Angioguard and Abciximab, neither Angioguard nor Abciximab. Prior to administration of Abciximab or placebo, the systolic blood pressure should be ≤160 mmHg. If the systolic blood pressure is > 160 mmHg, administer anti-
hypertensive medications, under the direction of the enrolling physician, to achieve a systolic blood pressure $\leq 160$ mmHg.

- When appropriate, place only an arterial sheath for vascular access
- Puncture only the anterior wall of the artery or vein when establishing vascular access
- The use of a through-and-through technique to identify the vascular structure is strongly discouraged.

Heparin and Abciximab administration

After sheath insertion 50 u/kg of heparin will be administered. An ACT should be measured 5 minutes after heparin administration with additional heparin given if the ACT is less than 250 seconds. The target ACT is defined as 275 seconds with a target range of 250 to 300 seconds. A bolus of 0.25 mg/kg Reopro (or placebo) will also be administered. Immediately thereafter begin the infusion of Reopro (or placebo) at 0.125 $\mu$g/kg/min (up to a maximum of 10 $\mu$g /min) for 12 hours continuous infusion. Study drug kits will contain 5 mL vials of Abciximab (2 mg/mL) or placebo. Study agent should be inspected visually for particulate matter prior to administration; study agent containing visibly opaque particles should NOT be used. Study agent for bolus injection is to be prefilled through a 0.2 or 0.22 micron low-protein-binding filter (Millipore SLGV025LS or equivalent) before injection. All continuous infusions should be filtered either upon admixture using a 0.2 or 0.22 micron low-protein-binding filter (Millipore SLGV025LS or equivalent) or upon administration using an in-line 0.2 or 0.22 micron low-protein-binding filter (Abbott #4524 or equivalent). As with all parenteral drug products, aseptic procedures should be used during the administration of study agent. Whenever possible, study agent should be administered in a separate intravenous line; no other medications should be added to the infusion solution.

For the continuous infusion, the study agent (placebo or Abciximab) will be diluted in normal saline according to site-specific instructions. A nomogram for study agent infusion rate, appropriate to the infusion solution concentration used at a given site, will be provided in the Study Reference Manual. Care must be taken to use the appropriate nomogram for the infusion solution concentration used.

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Study agent vials should be stored at 2°C to 8°C. Study agent should not be frozen or shaken. Once prepared, the infusion solution is stable at room temperature for up to 30 hours.

Infusion of study agent must be permanently terminated if the patient develops signs or symptoms suggesting a severe allergic reaction. Epinephrine, dopamine, theophylline, antihistamine, and hydrocortisone should be available for immediate use in the event of an anaphylactic reaction. The Abciximab or placebo infusion will continue for a total of 12 hours. The ACT should be 200 seconds or greater, prior to manipulation within the renal artery.

Interventional procedure

If a pressure gradient is measured, only a 0.014 inch pressure sensing guidewire may be used and simultaneous pressures should be obtained from the aorta. Pressure gradients with catheter intubation are prohibited.

Ten minutes after administration of the Abciximab bolus they may undergo implantation of a renal artery stent. The artery will be engaged with the guiding catheter, then an Angioguard XP Short Tip advanced across the lesion and deployed just proximal to the first bifurcation. An angiogram, with contrast injection, should be performed to document the position of the Angioguard XP Short Tip prior to performing the required pre-dilatation of the lesion. **Note:** The Angioguard XP Short Tip should be used in accordance with the Instructions for Use of this device (Appendix 2). If difficulty is encountered crossing the lesion with the Angioguard XP Short Tip device, pre-dilatation may be utilized using an undersized balloon. The following guidelines are recommended for selecting basket size, stent size, and identifying the minimum distance that must be present from the aortic ostium to the first bifurcation, in order to achieve distal protection:
Initially the lesion will be crossed with the Angioguard XP Short Tip, if the patient is randomized to use of this device. Pre-dilatation of the lesion may be performed with an undersized balloon if the Angioguard does not cross the lesion. After positioning of the Angioguard or guidewire, pre-dilatation must be performed prior to treatment with the stent. Use of a monorail balloon is recommended for pre-dilatation, although not required. After performing the required pre-dilation, stenting will be performed with 12mm or 15mm Genesis stent on the Aviator balloon according to accepted technique. The goal of stent treatment will be to achieve 1:1 sizing of the treated lesion with a normal appearing segment of vessel distally. However, if, in the investigator's opinion, there is a reasonable risk of renal perforation or rupture, with symptoms of back pain or other symptoms, less than 1:1 stenosis expansion is acceptable. The residual stenosis should be <50% with a >30% reduction in stenosis severity associated with normal renal arterial blood flow (TIMI grade 3). At the completion of the procedure the Angioguard XP Short Tip will be removed according to accepted practice utilizing the capture sheath. Other than a pressure gradient (with a 0.014 inch wire), no other manipulation may be performed in the renal artery without an Angioguard in place. The Angioguard XP Short Tip will not be flushed but will be submitted to the core pathology lab for analysis.

**Sheath removal**

Upon completion of the procedure, a femoral angiogram is required if a closure device is to be utilized for hemostasis. It is recommended that a systolic blood pressure of ≤ 160 mmHg should be achieved prior to sheath removal. If the systolic blood pressure is > 160 mmHg, consider use of intravenous anti-
hypertensive agents to achieve blood pressure control at the time of, and in the 12 hours after, sheath removal. Following are recommendations for post-procedural patient care for patients receiving manual or mechanical compression for hemostasis. When a closure device is utilized, these guidelines may be utilized, at the discretion of the investigator. Institutional guidelines may also be utilized.

Post-procedure patient care recommendations for patients receiving manual or mechanical compression for hemostasis:

Prior to sheath removal
- Check sheath insertion site and distal pulses of affected leg(s) q 15 minutes x 1 hour, then hourly x 6 hours
- Maintain complete bed rest with head of bed < 30 degrees
- Maintain affected leg(s) straight via sheet tuck method or soft restraint
- Medicate for back/groin pain as necessary
- Educate patient on post-PTCA care via verbal instructions and handbook

Sheath removal (no closure device)
- Achieve a systolic blood pressure ≤ 160 mmHg.
- Do not remove sheath within 2 hours after discontinuation of heparin
- Check aPTT or ACT prior to sheath removal: do not remove sheath unless aPTT < 50 seconds or ACT < 170 seconds
- Apply pressure to access site for at least 30 min following sheath removal, using either manual compression or a mechanical device
- Apply pressure dressing after hemostasis has been achieved

Post-sheath removal
- Maintain a systolic blood pressure ≤ 160 mmHg.
- Check groin for bleeding/hematoma and distal pulses q 15 minutes x one hour or until stable, then hourly
- Continue complete bed rest with head of bed ≤ 30 (and affected leg(s) straight for 6-8 hours following sheath removal, or 4 hours following discontinuation of heparin, whichever is later

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• Remove pressure dressing prior to ambulation
• Continue to medicate for discomfort
• Re-initiation of heparin following early sheath removal is discouraged; however, if this is considered clinically necessary, an infusion of heparin may be started a minimum of 4 hours after sheath removal at a rate of 10 U/kg/hr without a preceding bolus. The heparin infusion should be titrated every 6 hours, as needed, to maintain a therapeutic aPTT between 50 and 70 seconds.
• Patients should be medicated for groin and back pain as necessary.

Management of Access Site Bleeding
• Lower head of bed to 0 degrees
• Apply manual pressure/compression device (e.g., Femstop or Stasis button) until hemostasis achieved
• Change pressure dressing as needed
• Obtain stat CBC with platelets
• If heparin infusing, obtain stat aPTT and adjust heparin per M.D.
• Maintain intravenous access if sheath has been removed
• Contact M.D. and transfuse according to physician guidelines

In hospital laboratory testing
Immediately post procedure a blood and urine sample will be obtained for the core laboratory. At 12 and 24 hours following the procedure a blood sample will be collected to assess measure serum LDH, complete blood count, and a sample to send to the core lab. With the 24 hour collection a creatinine determination will be performed. Two hour urine collections will also be performed between hours 10 and 12, and hours 22 to 24, and submitted to the core lab for analysis and urine protein electrophoresis. At the Medical College of Ohio site only, all core lab blood draws will include a whole blood sample for flow cytometry and whole blood aggregometry.

Platelet Counts
If the platelet count falls below 100,000 cells/µL, with a decrease of
at least 25% from the baseline value is obtained in this sample, additional platelet counts are recommended to exclude an artifactually low platelet count. Samples for these additional platelet counts should be drawn in 3 separate tubes, one containing EDTA, one containing citrate, and one containing heparin; a stat platelet count should be performed on each tube. In addition, a smear should be prepared from fresh blood (i.e., no added anticoagulants) from a fingerstick or a syringe needle for a platelet count done with a phase microscope. An artifactual fall in platelet count is diagnosed if the platelet counts in the citrate or heparin tube and on the smear are within normal limits. If the fall in platelet count is not artifactual (i.e., if the platelet counts in all three tubes and on the smear are below 100,000 cells/μL with a decrease of at least 25% from baseline), the study agent should be discontinued immediately and platelet count appropriately monitored until it returns to normal.

If a patient's platelet count is < 50,000 cells/μL, heparin and aspirin should be discontinued if the clinical situation permits or requires. For those patients with platelet counts of 20-50,000, platelet count should be rechecked every 6 hours. If vascular hemostasis or hemorrhage is an issue, platelets should be transfused immediately. If a patient's platelet count drops below 20,000 cells/μL, it is recommended that platelets be transfused to maintain a platelet count greater than 20,000 cells/μL.

Management of Serious Bleeding and Emergency Surgery

In the event of serious uncontrolled bleeding or the need for emergency surgery, study agent should be discontinued. Decisions regarding discontinuation of heparin should also be made on clinical grounds at the discretion of the investigator. In the majority of patients, bleeding time returns to < 12 minutes within 12 hours and platelet aggregation returns to ≥ 50% of baseline within 48 hours following discontinuation of Abciximab. The administration of platelets may reverse the platelet inhibitory effects of Abciximab and should be considered in any patient with clinical evidence of serious bleeding.

Platelet transfusions may also be of benefit in patients with serious bleeding who have received aspirin even if no Abciximab has been administered. A decision
to administer platelets should be based on clinical judgment, taking into account
the site and severity of bleeding and the time since discontinuation of Abciximab. In
the case of documented or suspected CNS hemorrhage, platelet transfusions are
strongly recommended regardless of treatment assignment. If a decision is made
to administer platelets, it is recommended that 10 units of platelets be given
initially. If platelet function does not return to normal or cannot be measured, repeat
platelet transfusions should be considered within 1-2 hours if there is evidence of
progressive CNS hemorrhage or uncontrollable gastrointestinal, genitourinary,
or retroperitoneal hemorrhage.

Transfusion Guidelines

The following guidelines are adapted from the American College of Physicians
Clinical Guideline: Practice Strategies for Elective Red Blood Cell Transfusion

Guidelines for Transfusions in Asymptomatic Patients

- Assess the patient's intravascular volume status. All asymptomatic patients
  should be normovolemic. Normovolemic anemia (hemoglobin 7-10 g/dL [70-
  100 g/L]) can be well tolerated in asymptomatic patients.
- If hypovolemic, intravascular volume of patients should be adequately restored
  with crystalloids.
- In asymptomatic normovolemic patients, transfusion is not indicated unless a
deterioration in vital signs is seen or unless the patient develops signs and
symptoms, as described below.
Guidelines for Transfusions in Symptomatic Patients

If the following signs and symptoms occur: syncope, dyspnea, postural hypotension, tachycardia, angina, transient ischemic attack

- Use crystalloids to replace intravascular volume.
- If symptoms persist after volume repletion, the patient should receive transfusion(s) with autologous blood (if available).
- If autologous blood is not available, the patient should receive transfusion(s) with homologous blood on a unit-by-unit basis to relieve symptoms.

Remember: One unit may be sufficient.

Blood Pressure Assessment

Systemic hypertension is defined as blood pressure ≥ 140 systolic and /or 90 diastolic mmHg (59). Baseline and follow-up blood pressure will be monitored during the study utilizing seated office blood pressure measurements, in the same arm for all visits. The arm recorded in the baseline visit should be utilized for subsequent measurements. These will be performed in a quiet room, after five minutes seated, and will be measure in triplicate with an oscillometric automatic blood pressure device or a mercury sphygmomanometer. Measurements will be obtained at baseline and before the 1 and 6 month office visits.

Stenosis Assessment: Angiographic and Hemodynamic

Angiographic success will be defined as a reduction in stenosis severity of ≥ 20% with a residual stenosis of < 30% as determined by digital caliper measurement. Restenosis on follow-up angiography, performed only if clinically indicated, will be defined as a narrowing of ≥ 50% by digital caliper measurement. Stenosis measurement technique by digital caliper involves comparison of minimal luminal diameter within the stenosis to a normal reference segment (80). For ostial stenoses the reference segment is considered to be a normal-appearing distal segment unaffected by post-stenotic dilatation. For non-ostial disease the reference segment will be measured proximal and distal to the stenosis in normal-appearing portions of the vessel. Pressure gradients will be measured with a
0.014" pressure measuring guidewire distal to the stenosis, placed through a more proximal 7 or 8 french guiding catheter (74).

Renal Function Assessment

At each visit blood urea nitrogen and serum creatinine concentrations will be measured. Baseline renal insufficiency is defined as a baseline serum creatinine ≥1.6 mg/dl.

At baseline and at 1 month the GFR will be assessed in the affected kidney utilizing the methods described in Appendix 1. Following is a brief summary of the methods. The iohexal clearance and 99mTc-diethylenetriamine pentaacetic acid (DTPA) scanning to assess the percent function of GFR for the affected kidney. Antihypertensive medications will be continued at the time of renal function assessment.

Iohexol Clearance

Anti-hypertensive medications will be continued during the baseline evaluation with the exception of diuretics which will be held the morning of GFR assessment. All patients will undergo an overnight fast and avoid the following drugs for 7 days prior: nonsteroidal anti-inflammatory agents (except aspirin), cimetidine, ranitidine, and trimethoprim. In addition, each patient will be instructed to drink ≥1 liter of water the day prior to the test and an additional 500 mL of water on the morning of the test. Subsequently, they will have their baseline office seated blood pressure determined. Renal function will be assessed that morning by iohexol clearance. Iohexol was selected as the method of choice for the following reasons: it can be measured centrally at a core laboratory, is accurate and reproducible (Gaspari F 1998), obviates need for urine collection, and is not radioactive.

Results of a local determination of creatinine (Cr) are used to derive a simple Cockcroft-Gault (CG) estimation of Cr clearance. Plasma iohexol is determined by HPLC (Gaspari F 1998). The plasma clearance of iohexol, calculated according to the Brochner-Mortensen formula (Brochner-Mortensen J 1972), will be used as the measure of glomerular filtration rate, and will be
standardized to body surface area. Commercial controls will be fortified with 2 levels of iohexol (low and high concentrations), assayed, aliquoted and stored frozen. Two controls will be run with each batch of patients. Stock standards will also be frozen. In addition, on a monthly basis, 2 patients will be reanalyzed to detect problems with the assay. Patients that have an adverse reaction to iodine-containing contrast media are excluded from study. If a patient develops a contrast reaction after enrollment, the GFR at termination will be determined with a 24 hour urine and calculated Cr clearance.

Placement of an IV catheter is synchronized with the routine fasting phlebotomy to avoid additional venipuncture. Verify the patient is not pregnant. Administer 5 mL of iohexol (Omnipaque) by slow IV push but within 2 minutes. Flush the catheter with 10ml of normal saline. The IV line may be converted to a heparin lock or saline IV line, thus avoiding further venipuncture. Note the exact time injection is completed and use the same clock for timing throughout the procedure. Zero time is the end of this bolus infusion. Blood samples are drawn at 2.5, 4 hour if the estimated Cr clearance is ≥ 50 mL/minute and at 2.5, 4, and 6 hours if it is < 50 mL/minute (Gaspari F 1998). It is extremely important that blood samples be drawn at the scheduled time. Blood samples should be 10 mL each and put into 2 green top plasma separator tubes. If any sample is drawn beyond the scheduled time, record the actual drawing time; the next drawing time should still be calculated from time 0.

99mTc-Diethylenetriamine Pentaacetic Acid (DTPA)

The same day as the lohexol clearance the patients will undergo DTPA and dynamic renal scintography (DRS) utilizing the most current and accurate methods as adapted from Steinmetz et al (84) utilizing methods outlined in Appendix 2. The only additional preparation for the DTPA is well hydration with at least 3 glasses of water prior to the test. The patient will be asked to hold any diuretic medication the morning of the exam.
Bleeding Classification

Bleeding is to be classified as major, minor, or insignificant, employing the TIMI Study Group criteria for bleeding. To account for transfusion, hemoglobin and hematocrit measurements will be adjusted for any packed red blood cells (PRBCs) or whole blood transfused between the baseline and post-treatment hemoglobin/hematocrit measurements. The number of units of PRBCs and whole blood combined will be added to the change in hemoglobin. Three times the number of units of PRBCs and whole blood combined will be added to the change in hematocrit. Bleeding that meets the criteria below for major or minor bleeding, but is associated with CABG, will be considered separately from other bleeding.

Major Bleeding

Major bleeding is defined as either 1) intracranial bleeding or 2) bleeding associated with a decrease in hemoglobin greater than 5 g/dL or, when hemoglobin is not available, a decrease in hematocrit greater than or equal to 15%, or 3) bleeding associated with need for transfusion.

Minor Bleeding

Minor bleeding is defined as any of the following: 1) spontaneous gross hematuria or hematemesis; 2) observed blood loss (spontaneous or nonspontaneous) with a decrease in hemoglobin greater than 3 g/dL (but < 5 g/dL) or, when hemoglobin is not available, a decrease in hematocrit greater than or equal to 10% (but < 15%); or 3) a decrease in hemoglobin greater than 4 g/dL (or, when hemoglobin is not available, a decrease in hematocrit < 12%) with no bleeding site identified despite an effort to find one.

Insignificant Bleeding

Insignificant bleeding is defined as blood loss not meeting criteria for major or minor bleeding.
General Nursing Care

Interventions to prevent minor bleeding

AVOID:

- Unnecessary arterial and venous punctures
- Intramuscular injections
- Routine use of urinary catheters
- Noncompressible IV access sites (i.e., subclavian or jugular veins)
- Nasotracheal intubation and nasogastric tubes
- Frequent phlebotomies

DO:

- Use saline or heparin lock for blood drawing
- Document and monitor puncture sites
- Maintain bleeding precautions
- Provide gentle care when removing dressings
- Check stools and urine for occult blood

Patient follow-up

Follow-up visits will be scheduled in the following intervals: one and six months from the date of randomization. At the 1 month follow-up (def: within 1 wk before or 2 wk after date of procedure) they will have their baseline office seated blood pressure determined. The blood urea nitrogen, creatinine, lactate dehydrogenase, and a serum sample for core lab analysis will be collected at the time of patient arrival. The patient will also be asked to hold their urine for two hours. This specimen will be collected and utilized for the following: immediate urine dipstick at the study site, and preparation of a sample to be frozen and shipped to the core lab for analysis and urine protein electrophoresis. Renal function will be assessed that morning by the lhexol clearance followed by dynamic renal scintography with DTPA. The 6 month visit up (def: within 1 month before or after date of procedure) will be for clinical assessment only at which time the only additional measures will include the blood pressure determination and serum creatinine determination.

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Statistical Plan

Comparisons between groups will be made using ANOVA, or non-parametric tools if the data is not normally distributed. Paired comparisons will be utilized to assess differences between baseline and follow-up conditions within groups. The following analyses will be performed:

- Effect of Angioguard XP Short Tip on 1 month affected kidney gfr
- Effect of Abciximab on 1 month affected kidney gfr
- Effect of combination therapy Angioguard and Abciximab on 1 month affected kidney gfr
- Retrieval of atheromatous material with Angioguard
- Effect of Abciximab on retrieval of material with Angioguard
- Effect of Angioguard XP Short Tip on urinary markers of inflammation or injury
- Effect of Abciximab on urinary markers of inflammation or injury
- Effect of Abciximab on:
  - Access site complications
  - TIMI bleeding
  - Incidence of peri-nephric hematomas or renal perforation

The primary efficacy analysis will be the comparison of the control group (no Abciximab and no Angioguard) with the combination of Abciximab and the Angioguard XP Short Tip on the change in affected kidney GFR between the baseline and 1 month examinations. This analysis will include only randomized patients and will be assessed in per-protocol fashion, where only patients actually receiving their assigned treatment, utilized in accordance with the protocol, will be analyzed for the efficacy analysis using appropriate statistical techniques. The safety analyses will include the roll-in phase subjects and the randomized subjects.
Data Management

All data will be recorded on pre-printed data collection forms, according to accepted practice, by study personnel in legible manner utilizing black ink. All CRF's should be reviewed, signed and dated by the PI. When corrections are necessary, these should be made by crossing out the original entry with a line and writing in the correct information beside the original entry. These corrections require the date and signature of the person making the correction. Relevant copies of identifiable source documentation will be kept with the study records at the study sites. Source documentation includes, but is not limited to, the following:

- Hospital or office records of patient visits, admissions, or treatments
- Medical history and physical examination information, performed by the coordinators, PI, or the PI’s designate
- Laboratory results
- Drug and device logs

Completion of data collection forms will be requested within 24 hours of a study event. All forms will be sent by fax to the data center for data entry. Every 2 months sites may be audited by a study nurse (see Site Monitoring).

The database and entry screens for this project will be developed using FileMaker Pro® databases. To minimize errors all entry fields will be programmed to detect inconsistent and invalid data. Specifically, data will be checked for invalid codes, values that are out of range, and invalid dates and skip patterns. The data will be audited for incomplete information. A list will be generated that displays patients with incomplete data and/or a pending follow-up within 24 hours after their expected one month or six month visit. The patient will be removed from the list once the data for the follow-up is entered or the incomplete information is provided. All data, double entered into the database, will be verified against the original forms. This database will be converted to a Statview® or SAS data set for analysis.

All data will be kept at a locked and secured site. Entry will be restricted to study personnel only. Access to the computerized data will be limited to the appropriate study personnel and will be stored on a dedicated computer without network access. In order to logon to the computer each designated user will have their own username and password. The database itself will have a two-tiered
system of security. When initially accessing the database a password will be required which grants editing, record creation and other access privileges. The second level of security will require a username and password before patient data can be entered or viewed. This will also provide tracking of activity by a particular user in a patient’s record. Only the database administrator will have access to the data structure, programming, data exporting and record deletion features of the database. The computer will be routinely backed up and the media will be stored off-site at a secure facility. Study documents will be kept in strict confidence. Use of study material will be limited to those items listed in the protocol and on the informed consent form.

Randomization

The first 2 subjects from each center will be treated with the Angioguard XP Short Tip, in addition to the Genesis stent. These subjects will not be randomized. Following is the randomization plan for the subsequent 100 subjects:

Randomization schedules have been generated from computer based pseudo-random number generators with the following allocations:

- 1/2 to Angioguard XP Short Tip, 1/2 to no Angioguard XP Short Tip
- 1/2 to Abciximab, 1/2 to no Abciximab

To limit confounding, randomization will be stratified by the following factors: renal insufficiency (Cr ≥1.6 mg/dL) and treatment site. The personnel involved in patient care will not be aware of the randomization schedule or have access to it. Each site will be provided with two sets of sealed sequentially ordered randomization envelopes, one for patients with normal renal function and one for patients with renal insufficiency. Upon randomization a unique study number and group assignment will be allocated to the patient. Selecting a randomization envelope out of sequence or from the wrong renal function set are protocol violations necessitating notification of the Clinical Coordinating Center, completion of an entry in the protocol deviation log, and discarding the envelope that should have been used. Randomization should proceed in sequential order from the point where the violation occurred. If more than one envelope was skipped the Clinical
Coordinating Center will instruct the site on how to remedy the sequencing in order to preserve balanced allocation to treatment groups.

**Monitoring of adverse events**
At every patient assessment the study site will assess the patient for the occurrence of adverse events. An adverse event is any untoward medical occurrence in a patient. It does not have to have a causal relationship with the study treatment. It can be any unfavorable sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of the study agent(s). Any serious unexpected adverse event should be reported to the coordinating center by telephone, fax or written communication within 24 hours. Completed adverse event forms should be forwarded to the coordinating center within 5 additional calendar days. All such serious and unexpected adverse events will be reported to the FDA and to the MCO IRB at the time we are notified. Additionally, the sites will be notified of such events and they will be required to notify their IRB.

Adverse events should be graded in the following manner:

- **Seriousness**
  - Serious: death, life-threatening, requires inpatient hospitalization or prolongation of hospitalization, results in persistent or significant disability or incapacity, results in a congenital anomaly
  - Not serious: adverse event not resulting in the above

- **Severity (Intensity):** mild moderate or severe

- **Expectedness:**
  - Expected Identified within the study protocol as a known complication of study treatment or as a known complication of medications or devices utilized within the patients treatment.
  - Unexpected: Any event that is not consistent with the nature or severity of information in the study protocol.

- **Relatedness to study drug or device:**
  - Not, unlikely, possibly, probably, definitely, not known (definition then reverts to possibly)

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Clinical outcome: recovered, not yet recovered, died
Frequency: single or multiple
Did adverse event result in surgery or procedure?

**Potential risks:**

<table>
<thead>
<tr>
<th>Potential Adverse Effects</th>
<th>Seriousness</th>
<th>Likelihood</th>
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</thead>
<tbody>
<tr>
<td>Bleeding</td>
<td>High</td>
<td>&lt; 5%</td>
</tr>
<tr>
<td>Bleeding from access site</td>
<td>Low</td>
<td>&lt; 5%</td>
</tr>
<tr>
<td>Blood vessel injury</td>
<td>Moderate</td>
<td>&lt; 1%</td>
</tr>
<tr>
<td>Pseudoanuerysm</td>
<td>Moderate</td>
<td>&lt; 5%</td>
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<tr>
<td>Permanent renal failure</td>
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<td>Permanent renal insufficiency</td>
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<td>Transient Renal Failure</td>
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<td>Renal infarction</td>
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<tr>
<td>Need for Surgery</td>
<td>Moderate</td>
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<tr>
<td>Death</td>
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<td>Loss of arm or leg</td>
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<td>Vessel Thrombosis</td>
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<td>Renal artery aneurysm</td>
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<td>Embolization of stent</td>
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<tr>
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**Other Risks**

**Elicited Immune Response**

Positive immune responses to Abciximab have occurred in a small portion of treated patients. Of 1232 patients evaluated for human antichimeric antibody (HACA) response after receiving Abciximab bolus plus infusion in the EPIC, CAPTURE, and EPILOG trials, 73 (5.9%) had a positive response. The majority of patients with a positive HACA response had a low titer response (< 1:1600). Results were consistent across the 3 trials.
Re-administration

There are only limited data on re-administration of Abciximab. Administration of Abciximab may result in HACA formation which may cause hypersensitivity reactions (including anaphylaxis), thrombocytopenia, or diminished benefit upon re-administration of Abciximab. Readministration of Abciximab to 29 healthy volunteers who had not developed a HACA response after first administration did not lead to any change in Abciximab pharmacokinetics or to any reduction in antiplatelet potency. However, results in this small group of patients suggested that the incidence of HACA response may be increased after re-administration. Re-administration to patients who have developed a positive HACA response after initial administration has not been evaluated in clinical trials. Patients with HACA titers may have allergic or hypersensitivity reactions when treated with other diagnostic or therapeutic monoclonal antibodies.

Thrombocytopenia

Among the 5461 patients randomized to placebo or Abciximab bolus plus infusion in the EPIC, CAPTURE, and EPILOG trials, 165 patients had a fall in platelet count to less than 100,000 cells/µL, 46 in the placebo treatment groups (2.03%) and 119 in the Abciximab treatment groups (3.73%). The difference in the incidence rate between the groups was 1.70%, with an upper limit to the 95% confidence interval of 2.58%. Among 2270 patients randomized to placebo, there were 11 reports of a fall in platelet count to less than 50,000 cells/µL, compared with 34 in the 3191 patients randomized to Abciximab, a difference in incidence of 0.58% (upper limit to the 95% confidence interval, 1.04%). The largest difference between placebo and Abciximab with respect to fall in platelet count was seen in the CAPTURE trial (1.26% in the placebo group had a fall in platelet count to less than 100,000 cells/µL vs 5.56% in the Abciximab group). Falls in platelet count were transient and typically occurred during the first few days of therapy. The number of patients who had a fall in platelet count to less than 50,000 cells/µL and serious, life-threatening or fatal adverse events was not increased in the Abciximab treatment groups (9/11 patients [81.8%] in the placebo groups and 9/34 patients [26.4%] in the Abciximab groups).
Bleeding

The major direct risk of Abciximab treatment is bleeding. The exclusion criteria are designed to prevent individuals from entering the study who are at increased risk of bleeding due to an underlying condition. In the EPIC trial, in which non-weight-adjusted doses of heparin were used, major bleeding not associated with CABG occurred in 10.6% of patients in the Abciximab bolus-plus-infusion group and 3.3% of patients in the placebo group. The most common site of bleeding was the vascular access site. Bleeding rates were reduced in the CAPTURE trial as the result of improved patient management guidelines and a recommendation to weight-adjust intraprocedural heparin doses that was made as the trial was ongoing; major bleeding not associated with CABG occurred in 3.8% of patients in the Abciximab group and 1.9% of patients in the placebo group. In the EPILOG trial, the incidence of bleeding was further reduced through the use of lower dose weight-adjusted heparin, weight-adjusted Abciximab infusion, and specific patient management and vascular access site guidelines (including a strong recommendation for early sheath removal). Major bleeding not associated with CABG occurred in 1.9% of patients in the Abciximab plus standard-dose weight-adjusted heparin group, 1.1% of patients in the Abciximab plus low-dose weight-adjusted heparin group, and 1.1% of patients in the placebo plus standard-dose weight-adjusted heparin group.

Because Abciximab inhibits platelet aggregation, caution should be employed when it is used with other drugs that affect hemostasis, including oral anticoagulants, non-steroidal anti-inflammatory drugs, dipyridamole, thrombolytic agents and ticlopidine. In the EPIC trial, there was limited experience with the administration of Abciximab with low molecular weight dextran. Low molecular weight dextran was usually given for the deployment of a coronary stent, for which oral anticoagulants were also given. In the 11 patients who received low molecular weight dextran with Abciximab, 5 had major bleeding events and 4 had minor bleeding events. None of the 5 placebo patients who were treated with low molecular weight dextran had a major bleeding event. Because of the potential for bleeding, treatment with Abciximab requires careful attention to all potential
bleeding sites including catheter insertion sites, arterial and venous puncture sites, cutdown sites, needle puncture sites, and gastrointestinal, genitourinary, and retroperitoneal sites.

**Surgical Risk**

An additional risk is posed in this patient population by the possible requirement for coronary artery bypass graft (CABG) surgery within the first few days after Abciximab administration. The risk associated with major surgery may be reduced by the administration of pooled human platelets before or during the procedure. In the EPIC trial, major blood loss (i.e., > 5 g/dL change in hemoglobin after adjustment for transfusion) among patients undergoing CABG during the index hospitalization was similar in the placebo group (24/33 or 72.7%) and the Abciximab bolus plus infusion group (26/33 or 78.8%); similar percentages of patients received transfusions of packed red blood cells or whole blood (60.6% placebo, 75.8% bolus plus infusion) and platelets (51.5% placebo, 48.4% bolus plus infusion) in association with CABG. In the EPILOG trial, the incidence of major blood loss among patients undergoing CABG during the index hospitalization was similar in the placebo group (19/26 or 73.1%) and the Abciximab plus low-dose heparin group (9/11 or 81.8%) but was higher in the Abciximab plus standard-dose heparin group (16/16 or 100%). Because fewer patients in the Abciximab groups underwent CABG during the index hospitalization, the incidence of major CABG-related blood loss in the overall EPILOG population was lower in the Abciximab groups than the placebo group.

**Other Risks**

No long term animal studies have been performed with Abciximab to evaluate its carcinogenic or mutagenic potential or its effect on the fertility of men or women. Abciximab may cause excessive menstrual bleeding and increased risk of uterine bleeding which may affect implantation of an ovum or cause abortion. It is not known whether Abciximab is excreted in human milk or absorbed systemically after ingestion; therefore, caution should be used when Abciximab is administered to nursing women.
Other risks, not associated with the investigational agents, in patients with renal artery stenosis and hypertension

In this population the other adverse clinical events seen include angina, unstable angina, myocardial infarction, arrhythmias, congestive heart failure, dizziness, syncope, rupture or occurrence of aortic aneurysms. As a consequence, the following procedures are often performed in this population: coronary artery bypass surgery, percutaneous coronary intervention, carotid endarterectomy, repair of aortic aneurysms.

Procedures for protecting against or minimizing potential risks to human subjects:

Prior to study initiation all investigators will be instructed on appropriate use of the Angioguard and Palmaz Genesis stent, and appropriate patient selection, in an effort to minimize the risk associated with the use of these devices.

To minimize risk associated with the use of Abciximab we have provided the investigators with protocols for concurrent administration of heparin, to insure adequate and not excessive anti-coagulation. We have also provided guidelines for management of hypertension beginning prior to the procedure and continuing for the duration of the study drug (or placebo) infusion. Finally, we will assess a platelet count at the end of study drug infusion so that, if thrombocytopenia is encountered, corrective action may be taken.

Appropriate angiographic technique will be utilized to expose patients to the least amount of contrast dye and radiation while providing high resolution angiograms. Additionally, the methods for nuclear studies have been described in detail within the attached appendix to insure the safety of the study and the quality of the data collected.

If women of childbearing age are candidates for participation, a pregnancy test will be performed. If they are not pregnant, participation will be offered if they are willing to utilize effective contraceptive methods through the course of the study. If they are unable or unwilling to do so, participation should not be offered.
Study site monitoring

Prior to enrollment of study subjects, the sponsor-investigator and monitor will hold a pre-investigation meeting to ensure that the investigators:

- Understand the investigational nature of the devices and drug and the requirements for device and drug accountability
- Understand the protocol
- Understand the requirements for a well-controlled study
- Understand and accept the obligations to conduct the study in accordance with FDA Part 812, 813, and other applicable regulations
- Understand and accept the responsibility to obtain informed consent in accordance with FDA and IRB regulations.
- Understand and accept the obligation to obtain IRB review and approval prior to study initiation and to conduct the study in accordance with IRB policies, including periodic review.
- Have access to adequate numbers of study subjects
- Have adequate facilities for study conduct
- Have sufficient time to conduct the study

The Principal Investigator will ensure that all necessary source documentation will be available to the study monitor. The study personnel will be available at these visits to correct deficiencies or resolve queries.

The study monitor will conduct site visits to ensure that:

- The facilities are acceptable for the purposes of the study
- The study protocol is being followed.
- Protocol changes have been approved by the IRB, and have been reported to the sponsor and or IRB.
- Accurate, complete and current records are maintained.
- Only the investigator and their delegated staff are carrying out agreed upon activities.
- During these visits the monitor will also review study records to ensure that:
- The information recorded is complete, accurate and legible

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• There are no omissions in the reports of specific data elements such as administration to any subject concomitant test drugs or devices or the development of an unreported adverse event.
• There are no missing visits or examinations noted in the reports.
• Subjects failing to complete the study, and the reason(s) for failure, are noted in the reports.
• Informed consent has been documented.

The monitor will maintain a record of findings, conclusions, and actions taken to correct deficiencies at each visit. The record will include:
• Date of visit
• Name of monitor
• Name and address of investigator that was monitored

Findings, conclusions, and actions taken to correct any deficiencies noted during the visit.

Data Safety Monitoring:
A Data Safety Monitoring Board, headed by Dr. Anand Mutgi, will review the safety results of the study and recommend termination, if necessary. Due to the small sample size, there will be no planned interim analysis for efficacy. Dr. Mutgi will be assisted by Dr. Modi, a nephrologist. Statistical support will be provided by Dr. Sadik Khudar. An interim safety analysis will be performed after enrollment of the first 50 subjects. Additionally, serious adverse events will be reported to the DSMB, as they occur.

The interim safety analysis will include the following:
• Procedures used for preparation of the report, including cutoff date
• List of participating centers and enrollment by center
• Number of subjects randomized, by treatment
• Distribution of patients by time of entry
• Frequency distribution of selected baseline characteristics: gender, race, age, Cr
• Enrollment of ineligible patients

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- Number of patients who did not receive assigned treatment
- Deaths, by treatment
- Serious adverse events, by treatment
- Unanticipated problems

Benefits to subjects:

In this trial all patients with renovascular hypertension will be treated with the Genesis stent. In addition, those randomized to the Angioguard XP Short Tip device or Abciximab may be protected against atheromatous or platelet embolization. This, in turn, may improve renal function, and possibly survival, after the procedure.

Benefits to mankind:

Currently, the most frequently utilized strategy for the control of renovascular hypertension is the placement of endovascular stents. However, such procedures may be complicated by worsening renal function. If either use of the Angioguard XP Short Tip device or treatment with Abciximab improve late renal function, this in turn may result in better late survival for patients.

Institutional Review Board
During the conduct of the study the investigator must:

- Understand and accept the obligation to obtain IRB review and approval prior to study initiation and to conduct the study in accordance with IRB policies, including periodic review.
- Obtain approval of Protocol changes from the IRB, and report such changes to the sponsor.

Site Discontinuation
If the sponsor discovers that an investigator is not complying with the signed study agreement, study protocol, IRB requirements, or FDA requirements, or conditions imposed by the IRB or FDA, the sponsor will promptly either secure compliance or discontinue shipment of the drug and devices to the investigator and terminate the
investigator's participation in the study. The investigator will also be required to return, or dispose of, any unused investigational material.

Regulatory Compliance
The study will be conducted in compliance with the protocol, Good Clinical Practice (GCP), the applicable regulatory requirements of the United States Food and Drug Administration (FDA), ICH guidelines, and local legal and ethical requirements.

Before the study is initiated at a study site, the following documents must be provided to the coordinating center:

- The original US FDA for 1572, Curriculum Vitae, and medical licenses signed by the Principal Investigator and sub-investigators.
- Institutional Review Board (IRB) membership list
- Written documentation of the IRB approval of protocol and informed consent document, with a copy of the IRB approved consent form.
- Laboratory certifications with current normal ranges for all laboratory tests conducted, per protocol, at the sites.
- Signed Investigator's Agreement.
- Financial disclosure information

All of the above, for the entire study period must be available to the coordinating center at study termination. In addition, the following must be on file.

- Completed Delegation of Authority Form
- Completed case report forms with source documentation
- Signed informed consent form(s) for each subject
- All IRB correspondence
- Copies of adverse event/SAE reports
- Study correspondence
- Study enrollment, Exclusion, Informed Consent logs
- Completed Drug and Device Accountability Records, Drug and Device Inventory Log and Returned Medication Inventory forms

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Drug and Device Logs
The drugs and devices will be shipped to the study sites. The quantities received must be verified and a record retained. A record of drug and device utilization must be maintained by the study site. Records should be maintained for the receipt, utilization, destruction, or return of study drugs and devices. The date of event, lot number, patients identification and study number should be recorded.
In the event of drug or device loss, theft or inadvertent contamination, a detailed description should be recorded. Upon study completion all unused material should be returned to the coordinating center, along with documentation for all utilized material, including copies of the drug and device log.

Record retention
Study documents should be kept for a minimum of 2 years after study completion. Should the study be utilized by the FDA for a marketing application, additional record retention at the study sites may be required.

Literature


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Appendix 1. Assessment of renal function.

Baseline DTPA Worksheet

99mTc-Diethylenetriamine Pentaacetic Acid (DTPA) protocol

Prior to the renal artery stenting the percent function of each kidney will be measured with DTPA. Renal depth will be calculated using direct ultrasound measurement in order correct for tissue attenuation. A syringe of 3 mCi DTPA will be imaged with a digital gamma camera (General Electric Star 3000i) and counted. Patients will be positioned over the camera in the supine position and receive the counted dose as an injection in their antecubital vein. A dynamic scan will be started in a 128 x 128 frame matrix for the next 30 minutes at the following intervals: 1) First minute at a rate of 2 sec per frame; 2) the remaining 29 min at 30 sec per frame.

Immediately following, one posterior and two lateral orthogonal (left and right) renal static images for 400 kilocounts will be taken. The injection site will be scanned to exclude subcutaneous infiltration and the empty syringe will scanned for residual activity.

GFR will be calculated individually for each kidney according to the Gate’s method from the background and attenuation-corrected DTPA uptake from 120-180 sec after tracer arrival to the kidneys. Background will be estimated using a semi-lunar region of interest (ROI) around the lower pole of the kidneys. Attenuation will be calculated using the renal depth measured directly from the orthogonal lateral views. The precision of measurement will be enhanced further by drawing a ROI around the lateral view of the kidney, finding the geometric center of this ROI using the computer, and finally, measuring the distance from this point to the posterior body surface along a line perpendicular to the table top. This method is preferred
because it gives a more accurate estimate of renal depth than the previously used Tonnesen regression formula (84).

**DTPA Renal Summary Scan Results***:

<table>
<thead>
<tr>
<th>DTPA GFR</th>
<th>% Contribution right kidney function</th>
<th>% Contribution left kidney function</th>
</tr>
</thead>
</table>

*Please attach the DTPA scan results to this worksheet*
ABSTRACT

Although there is interest in the use of renal artery stenting for renal dysfunction, the frequency, causes, and outcomes of acute injury from renal stenting are not well described. The aim of the current study is to determine the frequency of acute renal injury (defined as a 0.5 rise in serum Cr) 1 month after renal stenting, identify factors associated with acute renal injury, and determine whether renal injury is associated with late clinical events.