I-SPOT

Insights on Selected Procoagulation markers and Outcomes in stroke Trial

PROTOCOL

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I CLINICAL SITES

The ***I***nsights on ***S***elected ***P***rocoagulation markers and ***O***utcomes in ***S***troke ***T***rial (I-SPOT) is an ancillary study to the ***S***troke ***H***yperglycemia ***I***nsulin ***N***etwork ***E***ffort (SHINE) Trial. Temple University Hospital will serve as the first recruitment site. Additional sites will first be chosen from the Philadelphia Neurological Emergencies Treatment Trials (Phila-NETT) spoke hospitals that are participating in the parent study, SHINE. Moving forward all SHINE sites will participate in I-SPOT. A current list of participating sites will be posted on www.clinicaltrials.gov.

II STUDY TEAM ROSTER

Name	Role(s)
William Barsan	Advisory Committee
Gunter Boden*	Executive Committee
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III ABSTRACT

Activation of the tissue factor (TF) pathway of blood coagulation and fibrinolytic inhibition are associated with increased blood thrombogenicity; and increases in markers of blood coagulation levels may in part explain the high degree of poor neurological outcome and recurrence of stroke in patients with acute ischemic stroke (AIS). It has been shown that membrane-bound tissue factor procoagulant activity (TF-PCA) and plasma activated factor VII (FVIIa) and markers of thrombin generation, prothrombin fragment 1.2 (F1.2) and thrombin-antithrombin complexes (TAT), are markedly elevated after AIS[1]. While these markers are highest in patients with type 2 diabetes mellitus (T2DM) and hyperglycemia, the effect of blood glucose (BG) control on levels of blood coagulation markers and their relationship with stroke outcome is unknown. Therefore, a better understanding of the relationships between these and other markers of blood coagulation and clinical outcomes after stroke and how hyperglycemia control modulates these markers holds great promise for management of acute ischemic stroke.

The Insights on Selected Procoagulation Markers and Outcomes in Stroke Trial (I-SPOT): *Response to Insulin Administration and Blood Glucose Control* proposal is

designed to accompany the Stroke Hyperglycemia Insulin Network Effort (SHINE) clinical trial, a Phase III multicenter, randomized, controlled trial planning to determine the efficacy and validate the safety of glycemic control in stroke patients. The SHINE trial will recruit 1,400 AIS patients with Type II diabetes mellitus (T2DM) and hyperglycemia, each receiving 3 days of hyperglycemia control with intravenous (IV) insulin therapy or control therapy with subcutaneous (SQ) insulin. The I-SPOT trial will recruit 315 SHINE patients. Blood coagulation marker levels will be measured before and at 48 hours after the start of treatment. Baseline and temporal changes in biomarkers levels will be compared between SHINE treatment groups.

IV RESEARCH PROTOCOL

A. SPECIFIC AIMS

Aim 1: Compare the effects of strict hyperglycemia control with standard treatment of hyperglycemia on membrane-bound TF-PCA and markers of blood coagulation in T2DM patients after AIS.

Hypothesis: The decrease in levels of markers of blood coagulation will be greater in patients treated with IV insulin to reduce BG than in patients treated with SQ Insulin as the standard fashion.

Aim 2: Determine the relationship between levels of markers of blood coagulation and functional neurological outcome in SHINE treatment and control patients.

Hypothesis 1: The decrease in levels of markers of blood coagulation will be greater in patients with than without favorable (SHINE) outcome (defined as the baseline stroke severity adjusted measure of functional ability at 90 days after AIS).

Hypothesis 2: Hyperglycemia control modulates the relationship between blood coagulation levels and functional outcome in T2DM patients after stroke. Patients treated with IV Insulin for hyperglycemia control with favorable (SHINE) outcome will have greater decreases in blood coagulation levels than either IV Insulin-treated patients without favorable outcome or SQ Insulin-treated with or without favorable outcomes at 90 days after AIS.

B. BACKGROUND and SIGNIFICANCE

Over 750,000 people have strokes in the US annually. Approximately 40% percent of patients with acute ischemic stroke (AIS) are hyperglycemic at presentation. Patients with AIS, especially those with hyperglycemia, are at risk for both poor neurological outcome and recurrence of stroke. Determining the factors associated with these negative outcomes and discovering ways to modulate them is critical for treatment of AIS patients. It has been previously demonstrated that markers of activation of the tissue factor (TF) pathway of blood coagulation are elevated in AIS patients, particularly those with type II diabetes mellitus (T2DM).[1] Recognizing that AIS outcomes are worse in patients with hyperglycemia and T2DM[2], we hypothesize that the negative outcomes may be due to the effect of DM and/or hyperglycemia on blood coagulation and fibrinolytic inhibition. We

propose to study the effect of glucose levels and glucose regulation on the TF pathway in AIS patients and seek to define the relationship between glucose levels, markers of activation of TF pathway, and clinical outcome.

Tissue factor and blood coagulation mechanisms lead to the formation of clot, a process that can undermine treatment of acute stroke, worsen clinical outcome, and result in stroke recurrence. The overarching goals of this research are to study the relationships between markers of blood coagulation and the neurological outcome of patients with T2DM treated for hyperglycemia with IV or SQ insulin after AIS. Understanding the relationship between these markers and clinical outcome in diabetic patients with persistent hyperglycemia and those treated with insulin to control BG is important for two reasons. First, these studies will provide a potential mechanism for the harmful effects of hyperglycemia and of the potential benefits of BG lowering insulin therapy after stroke. Persistent hyperglycemia is known to be injurious after ischemic stroke and there is strong rationale for glucose lowering insulin therapy to improve clinical outcome after stroke. This research will reveal whether these effects can at least in part be explained by alterations in markers of blood coagulation measured early after AIS and after glucose lowering insulin therapy. Second, these studies will help reveal potential targets for and a rationale for the study of novel antithrombotic strategies. If one or more of these markers are associated with clinical outcome, it may provide a basis for developing novel therapeutic strategies to target the tissue factor/factor VIIa pathway of blood coagulation.

The ISPOT study has been expanded to include SHINE subjects treated with IV tPA. Enrollment of this subgroup will address an important area of interplay between blood coagulation mechanisms, hyperglycemia and insulin therapy in the context of AIS. These findings may have an impact on the development of treatment strategies in AIS, including on the role of aggressive measures to lower BG and the potential use of novel antithrombotic agents that may ameliorate some of the hemostatic changes after tPA for stroke.

The results of this study will have enormous impact on the stroke community as healthcare providers currently manage hyperglycemic acute stroke patients every day without adequate evidence of best therapy. Regardless of the results of this study, it will provide an understanding of the potential mechanisms of action of stroke therapy and guide acute stroke management across the US.

B.1. Diabetes and Blood Coagulation in Stroke

Diabetes and Hyperglycemia in Stroke. Clinical studies have shown a significant relationship between hyperglycemia and poor outcome after acute stroke.[3-6] Retrospective studies have shown that hyperglycemia is associated with a worsening of post-ischemic brain injury[7] and cerebral edema.[8] Even slightly elevated BG levels of 125 to 130 mg% have been associated with a longer hospital stay, higher mortality rate,[9,10] and increased infarct volume by MRI.[11] Acute hyperglycemia has been associated with hemorrhagic transformation of infarct in animal experiments[12] and in clinical reports.[13-15]

However, and important to the proposed studies, a rise in serum BG levels during or after acute stroke may promote coagulation by inducing the activation of the TF pathway[1] leading to worsened clinical outcomes and recurrent stroke.

Glycemic Control and Insulin in Stroke. In animal models, insulin reduces neuronal damage and can improve functional outcome, reduce the area of infarction and reduce mortality after an ischemic brain injury.[16-18] Hyperglycemia control has been shown to improve clinical outcomes in multiple conditions including AIS. Data of patients hospitalized with AIS with concomitant hyperglycemia was retrospectively reviewed. In 960 patients studied, hyperglycemia on hospital admission was associated with a higher mortality rate than euglycemia (OR 3.15, 95% CI 1.45, 6.85, p=0.004). Glycemic control (normalization of BG) was associated with an absolute mortality rate nearly seven times lower (p=0.0002) than that of patients with persistent hyperglycemia over 48 hours of hospitalization. Adjusted multiple logistic regression showed glycemic control to be a strong independent determinant of survival (OR 8.52, Cl 2.52, 28.77, p=0.001) after AIS.[11]

The mechanisms by which insulin and hyperglycemia control may protect against the deleterious effects of hyperglycemia in stroke are not known. Insulin has been shown to reduce ischemic brain damage when given immediately before or after experimental brain ischemia. The reduced free radicals, tissue acidosis, brain edema, hemorrhagic transformation, cytotoxicity and improved autoregulation attributed to glucose lowering are each presumed to contribute.[23-28] Insulin and glycemic control also protect against thrombogenesis after acute myocardial infarction by reducing plasma fibrinogen and FVIIa and reducing plasma TF and plasminogen activator inhibitor1 (PAI1) levels.[29,30] These observations suggest that the deleterious effects of hyperglycemia during acute brain ischemia may be at least partly mediated by these mechanisms and reversible by insulin and rapid correction of hyperglycemia.

Hyperglycemia and Diabetes mellitus as 'Procoagulant States'. The tissue factor (TF) pathway (also called the extrinsic pathway) is the primary physiologic mechanism of initiation of blood coagulation.[31,32] TF is a membrane-bound protein and binding of native coagulation factor VII (FVII) to TF facilitates the cleavage of a single peptide bond that converts native FVII to the activated form factor VIIa (FVIIa). The resultant TF-FVIIa complex then activates factors IX and X to factors IXa and Xa, respectively, leading to the formation of the prothrombinase complex and thrombin generation. In addition, Factor X can be activated to factor Xa by the factor IXa-VIII complex in the presence of phospholipids;[31,32] this mechanism amplifies the generation of factor Xa once coagulation is initiated. Factor Xa activates prothrombin to thrombin with the release of the prothrombin fragment F1.2, a process that requires factor V and phospholipid. Tissue factor pathway inhibitor (TFPI) rapidly inhibits the TF-VIIa complex, a process that requires factor Xa.

TF is highly expressed in atherosclerotic plaques and initiates coagulation and thrombus formation when the vessel wall is injured or plaques are fissured.[33] In addition, there is a circulating pool of TF in blood that is associated with cells and microparticles. This

circulating TF is biologically active and TF bearing microparticles are highly procoagulant.[33-39]

Elevated coagulation factors have been documented in patients with T2DM and in both normal, healthy patients and patients with T2DM during oral glucose loading.[40-48] Acute hyperglycemia during oral glucose tolerance testing increases markers of blood coagulation and thrombin generation in normal controls and patients with diabetes.[44,45] One study reported that raising BG and insulin for 24 hours in young, healthy volunteers activated the TF pathway. There was a marked increase in circulating TF-PCA, and plasma FVIII, F1.2, and TAT indicating a hypercoagulable state (Section C). Hyperglycemia or T2DM alone and especially in combination are associated with increased levels of TF-PCA,[40] plasma fibrinogen[46] and plasma and urinary fibrinopeptide-A levels[47,48] Moreover, blood from patients with T2DM and elevated circulating TF levels perfused over collagen coated surface has been shown to enhance thrombus formation.[49]

Another study found that TF-PCA and FVIIa levels correlated with BG levels during the first 48 hours after acute stroke[1] (Section C). While TF-PCA and FVIIa levels were much higher in stroke patients as compared with normal controls, patients with T2DM and hyperglycemia had much higher FVIIa levels than stroke patients with normal BG.[1] By activating the TF pathway of blood coagulation, hyperglycemia may intensify the relationship between blood coagulation and clinical outcome after stroke.

B.2. Blood Coagulation and Clinical Outcome in Stroke

Hypercoagulability in Acute Ischemic Disease and Stroke. There is evidence linking TF and plasma FVII levels to acute ischemic heart and brain disease. FVII/FVIIa levels are strongly related to fatal ischemic cardiac events.[50,51] One explanation for association with fatal events is that FVII levels influence the outcome at the time of plaque rupture (and TF release) through thrombin generation, fibrin deposition, platelet aggregation and thrombus formation. FVIIa leads to increases in plasma levels of factors IX and X activation peptides and prothrombin fragment F1.2.[52] In patients undergoing angioplasty or stent implantation, elevated whole blood TF-PCA present before the procedure predicted restenosis.[53]

Like in acute myocardial infarction, there is activation of coagulation system and thrombin generation in acute stroke. TF and VIIa levels are elevated patients with acute stroke.[54,55] Plasma F1.2 and TAT are elevated in stroke patients when compared with control and these elevations are sustained for at least 6 months after stroke.[56] Elevated TAT levels 24 hours after stroke are associated with higher mortality,[57] and high TAT, F1.2 or D-dimer (marker of thrombin generation and lysis of crosslinked fibrin) can predict which patients deteriorate or have stroke progression when assessment at day 3 was compared to baseline after stroke.[58] These findings suggest that increased thrombin generation may reflect more complicated and thrombogenic atherosclerotic lesions or a sustained hypercoagulable state that threatens cerebral reperfusion after AIS.

Targeting Peri Stroke Blood Coagulation to Enhance Clinical Outcome. Early thrombolytic therapy and successful reperfusion are central to stroke treatment to enhance clinical outcome. However, thrombin generation, impaired endogenous fibrinolysis, and vascular injury may be involved in failure to recanalize blood vessels and arterial reocclusion despite thrombolytic therapy.[59,60] Reocclusion after pharmacologic thrombolysis still remains a major issue in the clinical setting. In one study, intravenous (IV) tissue plasminogen activator (tPA) treatment of the occluded middle cerebral artery failed to achieve recanalization in 22% of patients.[59] In addition, there was a reocclusion rate of 34% among those who initially had a partial or complete recanalization.[60]

Trials of antithrombotic therapies to date have not proven effective in acute stroke patients, and they increase bleeding risk. Aspirin alone is of limited benefit[61,62] and a large metaanalysis of 22 trials showed that immediate anticoagulation with heparin, low molecular weight heparins, or heparinoids was ineffective to reduce death or dependency in acute stroke patients.[63] These studies, however, have not targeted patients likely to be at a high risk for progressing stroke and did not screen patients for increased risk for clot formation or thrombogenicity. Selecting subsets of patients at highest risk for deterioration and recurrent thrombosis to enroll in trials of antithrombotic therapies may enhance the safety and efficacy of these agents. There is a pressing need to develop novel strategies in this regard. An understanding of the temporal changes in coagulation factors, their relationships to the intensity of glucose lowering therapy and the clinical outcomes all of which are likely to come from the proposed studies will provide a rationale for renewed effort to apply novel or existing anticoagulants in carefully selected patients where the risk-benefit ratio becomes acceptable. These may include, for example, novel inhibitors at the level of TF and FVIIa. Aside from this, our studies will carefully document, in a multicenter randomized trial, the impact of insulin therapy on blood coagulation mechanisms and provide potential mechanisms for a beneficial effect of aggressive lowering of glucose, the hypothesis being pursued in SHINE patients.

C. PRELIMINARY STUDIES

C.1. Overview: Preliminary studies strongly supporting the aims of this proposal are the multiple studies from Drs. Gentile, Rao, and Boden. Drs. Rao and Boden have reported that circulating TF-PCA is elevated during combined hyperglycemia and hyperinsulinemia in healthy subjects, and is associated with elevations in plasma F1.2, TAT, and FVIII levels.[40] Patients with AIS had markedly elevated levels of whole blood TF-PCA and plasma factor VIIa, and that VIIa levels correlated with stroke severity. Even more striking was the finding that, in diabetic patients, both TF-PCA and FVIIa levels strongly correlated with blood glucose concentrations.[1] These studies serve as the basis for selection of the biomarkers to study in I-SPOT.

C.2 Preliminary Studies on Circulating TF-PCA The assay for TF-PCA in whole blood as described by Key *et al*[65] has been applied to make several observations. This assay measures membrane-bound and microparticle associated TF in lysed whole blood in a two-stage clotting assay where FX is activated to FXa by FVIIa. Unlike most

previous studies, which have measured TF antigen in plasma, this assay measures TF activity in cell membranes. It is important to note that TF is an integral membrane protein and, therefore, more relevant to measure in cell membranes.

Circulating Tissue Factor Activity Is Increased by Hyperglycemia and Hyperinsulinemia In Healthy Subjects.[40] To evaluate the effects of hyperglycemia and hyperinsulinemia on the TF pathway, healthy individuals were studied for 24 hr each with specific infusion clamps. Hyperglycemia and Hyperinsulinemia Group (HG+HI) subjects were infused with BG to maintain levels at 200 mg/dl. Euglycemia and Hyperinsulinemia Group (EG+HI) subjects were infused with insulin to maintain serum insulin levels at 1000 pmol/l while BG was maintained at ~ 100 mg/dL. Selective Hyperglycemia and Euinsulinemia Group (HG+EI) subjects were infused with BG (~200 mg/dl) and somatostatin to block endogenous insulin secretion. Placebo Group subjects received only saline. At 24 hours, whole blood Combined hyperinsulinemia and hyperglycemia and to a lesser degree selective hyperinsulinemia for 24 hours in healthy volunteers increased circulating TF-PCA, monocyte TF surface expression and mRNA, plasma thrombin generation, and coagulation factors VII and VIII activities, suggesting that the coagulation system had been activated. In addition, platelet CD40L and platelet-monocyte aggregates increased, indicating platelet activation. Somatostatin abolished these changes. Hyperinsulinemia, but particularly the combination of hyperinsulinemia and hyperglycemia, creates a prothrombotic state and may, in addition, be proinflammatory and proatherogenic by virtue of the actions of CD40L and TF. These studies showed for the first time that hyperglycemia and hyperinsulinemia and particularly the combination increase circulating TF, even in normal subjects, and induce a procoagulant and proinflammatory state.

Elevated Circulating TF-PCA and Procoagulant State in T2DM.[41,42] Several important findings relating T2DM, hyperglycemia, hyperinsulinemia, and especially the combination deserve attention and further study. First, it has been shown that circulating TF-PCA, [41] and plasma Factor VII coagulant (FVIIC), FVIIa, TAT and F1.2 levels are elevated in T2DM indicating a chronic procoagulant state. Second, we found that raising glucose and insulin levels in T2DM leads to large increases in TFPCA, TAT, and F1.2 above the already elevated basal levels. In these studies, we found that either raising glucose or raising insulin concentration for 24 hr significantly increased TFPCA above basal levels in patients with T2DM. However, raising glucose and insulin levels together resulted in dramatic increases of TFPCA levels associated with increases in TAT and F1.2. We concluded that the combination of hyperglycemia and hyperinsulinemia, common in poorly controlled patients with T2DM, contributes to a procoagulant state that may predispose these patients to acute thrombotic events.[42]

C.3. Elevated Circulating Procoagulant Factors and Markers of Thrombin Generation in AIS[1] During cerebral ischemia, TF-PCA and FVIIa contribute to enhanced coagulation by stimulating thrombin generation, fibrin deposition, platelet aggregation and thrombus formation. Thus, patients with diabetes and hyperglycemia may have worse outcome after stroke because of augmented activation of the TF pathway, particularly in association with chronically elevated coagulation factors.

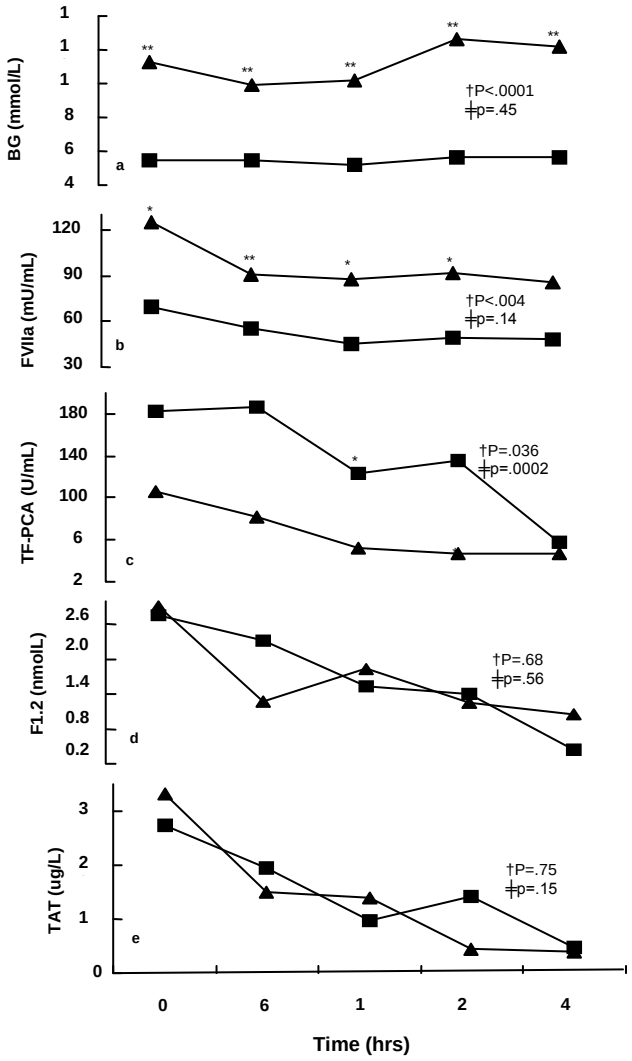


Figure 1. **BG, TF-PCA, FVIIa, TAT and F1.2 levels over time in patients with (-▲-) and without T2DM (-■-). Tests of fixed effects using two-way ANOVA for repeated measures to detect differences at each time point: †Group Effect; ╪Time Effect. Differences of Least Squares Means: *p<0.05, **p<.01 between groups include Dunn-Bonferroni adjustment for multiple comparisons. The graphs show that markers of blood coagulation levels decrease rapidly after stroke and that TF-PCA and FVIIA levels differ between patients with and without T2DM.**

The ***aims of this pilot study*** *were*: 1) to determine whether AIS was associated with changes in levels of TF-derived markers of blood coagulation and thrombin generation 2) to determine whether diabetes or hyperglycemia are associated with increased whole blood TF-PCA and plasma factor VIIa (the activated form of FVII), and 3) to determine the relationship between coagulation factor levels and F1.2, and TAT, both markers of thrombin generation after acute stroke. Blood glucose and circulating TF-PCA, FVIIa, F1.2, and TAT levels were measured at baseline (within 24 hours of stroke onset) and at 6, 12, 24, and 48 hours after baseline in 10 patients with DM and 11 nondiabetic patients.

Results: The average duration of symptoms was 9.2 ± 6.8 (range: 2-24) hours and average NIHSS was 6.6 ± 2.6 (range: 2-13). All patients had MRI evidence of acute or subacute stroke. Diabetic and nondiabetic patients were similar in terms of age, gender, baseline blood pressure, cell blood count, electrolyte studies and in aspirin or clopidogrel use before stroke or during the subsequent hospitalization (p>.05). Stroke symptom duration averaged 10.8 ± 7.4 hrs in diabetics and 7.82 ± 6.4 hrs in controls (p=NS). There was no difference in baseline NIHSS scores between diabetic and nondiabetic patients (7.6 ± 3.0 and 5.6 ± 1.8, respectively, p=0.09). However, at 48 hours, NIHSS was significantly higher in diabetic (9.25 ± 4.3) than nondiabetic patients (3.0 ± 1.6, p=0.019). *High levels of Markers of Blood Coagulation Measured early after AIS:* Whole blood TF-PCA levels were 8.5 fold higher in patients presenting with AIS (169 ±

xx U/mL) than healthy control subjects (20.7 ± 10.0 U/mL). Similarly, FVIIa levels were higher in patients after acute stroke (92 ± xx mU/mL) than in healthy subjects (64.2 ± 23 mU/mL, p=0.002).

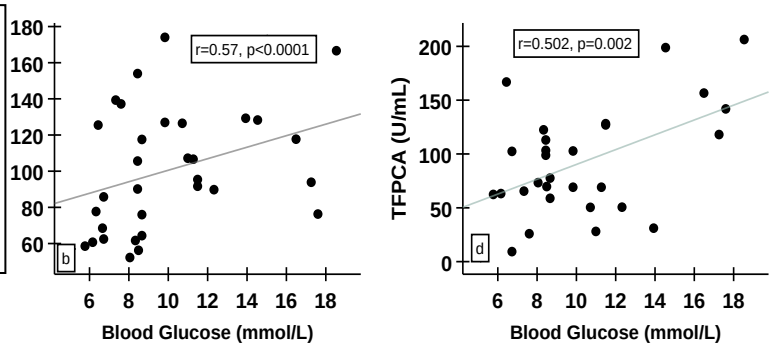
Figure 1 shows the mean blood glucose (BG), TF-PCA, FVIIa, TAT, and F1.2 levels at baseline and over the subsequent 48 hours in stroke patients with (-▲-) and without T2DM (-■-). As expected, diabetic patients had higher BG levels than nondiabetics at baseline and at each time point studied ($p \leq 0.01$, Fig 1a).

Markers of Blood Coagulation over 48-hours after stroke. TF-PCA levels fell from baseline over 48-hours after stroke presentation in both diabetic and nondiabetic patients (p=0.0002, time effect) yet remained substantially higher than in healthy control subjects (p=0.001). Likewise, FVIIa elevations persisted over time and levels were consistently higher among diabetic patients at 6, 12, and 24 hours ($p \leq 0.03$, Fig. 1b) as compared with nondiabetic patients. TAT and F1.2 levels fell to near normal levels over the first 48-hours after stroke.

Blood Coagulation Factor VIIa after stroke is higher in patients with than without T2DM. Plasma FVIIa was much higher in diabetic (124.4 ± 35.2 mU/mL) than in nondiabetic (69.7 ± 33.3 mU/mL) patients at baseline; and, levels remained higher in diabetic patients at 6, 12 and 24 hours than nondiabetic patients after stroke (Fig. 1c). FVIIa levels at 48-hrs in T2DM patients (86.0 ± xx mU/mL) were still substantially higher than in nondiabetic patients (48.3 ± xx mU/mL). There were no differences in F1.2 or TAT levels between the two groups at baseline or over 48 hours (Figs 1d and 1e). However, among diabetic patients, FVIIa levels correlated with both F1.2 (r =0.51, p=0.001) and TAT (r=0.62, p<0.0001).

FVIIa and TF-PCA levels in diabetic patients are related to BG levels after AIS. In diabetic patients both FVIIa and TF-PCA levels were positively related to BG (Figure 2). There were no relationships between BG and F1.2 or TAT levels. However, FVIIa correlated with plasma F1.2 (r =0.34, p=.002) and TAT (r=0.62, p<.0001). These findings suggest that AIS patients with diabetes and hyperglycemia have a more intense procoagulant state.

Figure 2. Relationship between BG and FVIIa (a) and TF-PCA (b) collected from patient with T2DM within 48 hours after AIS presentation. The graphs show that TF-PCA and FVIIA levels are positively correlated with blood glucose in diabetic patients after AIS.



C.4. Summary: The proposed studies follow directly from the pilot studies done by Drs. Gentile and Rao. Several preliminary results are particularly intriguing and deserve further consideration. One is the finding that hyperglycemia after stroke is associated with increased mortality.[9,10] Second is the finding that strict control of BG is associated with a >4-fold reduction in mortality.[11] Of key importance is the finding that AIS is associated with marked elevations in circulating TF-PCA and FVIIa, particularly in patients with T2DM and hyperglycemia,[1] because both circulating TF-PCA and FVIIa are thrombogenic and FVIIa is an important independent risk factor for acute events of atherosclerotic disease, including stroke and fatal coronary events.[50-55] These findings provide a strong rationale for the I-SPOT studies.

D. RESEARCH DESIGN and METHODS

D.1. Research Site Eligibility

I-SPOT is nested within the SHINE clinical trial, a single blind placebo controlled Phase III multicenter trial in which stroke patients with T2DM and hyperglycemia will be randomized to intravenous insulin infusion to control BG or standard management within 12 hours of stroke onset. I-SPOT is designed to accompany SHINE, from initiation of patient enrollment through study close-out. Only sites participating in the SHINE trial will be eligible to perform I-SPOT. Additional criteria are ability to process and store whole blood and plasma specimens until shipment to the Temple Thrombosis Center.

D.2. Patient Eligibility

A subset of patients enrolled in the SHINE study will be eligible for enrollment in the I-SPOT study.

SHINE Inclusion Criteria include: 1) Age 18 years or older; 2) Clinical diagnosis of ischemic stroke defined as acute neurological deficit occurring in one or more cerebral vascular territories; 3) Protocol treatment must begin within 12 hours after stroke symptom onset and is recommended, but not required, to begin within 3 hours after hospital arrival. If time of symptom onset is unclear or patient is awakening with stroke symptoms, the time of onset will be the time the patient was last known to be normal; 4) Known history of T2DM and BG >110 mg/dL **OR** admission BG ≥150 mg/dL in those without known DM; 5) Baseline NIHSS score 3-22; 6) Prestroke mRS above 0 for patients with an NIHSS score of 3-7. Pre-stroke Modified Rankin Scale score = 0 or 1 for patients with an NIHSS score of 8-22; 7) Able to provide a valid informed consent (self or legally authorized representative)

SHINE Exclusion Criteria include: 1) Known history of type 1 DM; 2) Substantial neurological, medical or psychiatric illness that would confound the neurological assessment or other outcome assessment; 3) Pregnancy or breast-feeding; 4) Other serious conditions that make the patient unlikely to survive 90 days; 5) Inability to follow the protocol or return for the 90 day follow up; 6) Renal Dialysis (including hemo or peritoneal dialysis); 7) Having received experimental therapy for the enrollment stroke.

IV tPA (up to 4.5 hrs) or IA tPA are allowed as are IA therapies including use of FDA cleared devices. Non FDA cleared devices are considered experimental and are excluded

Additional I-SPOT Criteria include:

Inclusion

a. Able to provide a valid informed consent to be in the study (self or their legally accepted representative)

Exclusion

a. Current or anticipated use of systemic anticoagulants, intra-arterial fibrinolytics (tPA) or mechanical thrombectomy

b. Known moderate or severe hepatic insufficiency (as defined by INR>1.5 if known or history of variceal bleeding or hepatic encephalopathy)

c. Prior or concurrent thrombotic or hypercoagulable condition (Antiphospholipid antibody syndrome; Antithrombin III, Protein C or S deficiencies; Congenital or Inherited Factor deficiencies; Sickle cell disease)

D.3. Recruitment and Enrollment

Eligible patients will be asked to participate in the I-SPOT trial as a part of the informed consent discussion for SHINE. This discussion is recommended to take place within 3 hours of arrival to the SHINE and I-SPOT participating site and within 12 hours of stroke symptom onset. After the patient or legally authorized representative agrees to participate and signs informed consent, the patient's blood will be drawn before study drug is administered. Patients who have received intravenous thrombolytics will have baseline blood samples drawn after tPA infusion has ended and before the start of study drug.

D.4. Sample Storage

Blood samples will be labeled with the subject's SHINE study number and the visit date. Blood samples will be stored in a locked laboratory at Temple University. Testing will be begin on these blood samples during the SHINE study enrollment period. Samples may be stored for an additional 3 years or longer after the data analysis is completed.

D.5. Post Enrollment Participant Contacts

As a participant in the I-SPOT trial patients will have blood drawn at baseline and 48 hours. On day 90 the patient will have an in-person visit as part of the SHINE trial. During this visit the mRS and QVSFS assessments will be performed. Patients who are unable to have the visit done in-person may have it done by phone or by proxy.

D.6. Risk Factor Management

The potential complications of venipuncture are few and relatively minor. The pain or burning associated with skin and vein puncture is usually mild and is generally completely relieved with a mild analgesic such as acetaminophen and acetaminophen based compounds. Aspirin, ibuprofen and other anti-inflammatory agent that act on platelets or on coagulation or fibrinolysis will be avoided. Swelling by edema or inflammation and hematoma will be treated with cool compresses, limb elevation, and acetaminophen or acetaminophen containing products for pain. Subjects who receive thrombolytics will be monitored closely for bleeding and hematoma at the venipuncture site. The incidence of local infection at the venipuncture site is very low and treated in standard fashion.

The confidentiality and privacy of study participants will be protected as data and records from these individuals will be available only to investigators and study specific research personnel. All patient identifiers will be removed from data shared with consultants and collaborators. Individual research participants will not be identified in any way in publications. The regulations outlined in HIPPA will be firmly followed and all patients will sign IRB approved study specific HIPPA forms. Treatment for any study related injury will be provided free of charge by local hospitals as is the norm. Data maintained in the study database will be accessible only to the project investigators, the study coordinator and the data coordinator using a password system for access.

Research study subjects will be monitored closely for complications and adverse effects on a regular basis over the study period.

D.7. Definition for Endpoints

The primary comparison is the change in biomarker between patients with favorable versus unfavorable functional outcome and the secondary comparison is the changes in biomarker levels between patients with versus without stroke recurrence at 90 days post stroke.

From the SHINE study, the definition of “Favorable” functional outcome is based on the baseline stroke Severity adjusted 90-day modified Rankin Scale (dichotomized mRS). “Favorable” is defined as an mRS score of 0 in subjects with baseline NIHSS of 3-7, mRS of 0-1 in subjects with baseline NIHSS of 8-14, and mRS of 0-2 in subjects with baseline NIHSS of 15-22. All investigators will be trained and certified in the performance of the mRS.

The secondary clinical outcome assessed, fatal and nonfatal stroke recurrence, is defined as a stroke that occurs during the interval between the index stroke for enrollment and the 90day assessment and will be identified by: 1) report of stroke on adverse events logs (i.e. neurological worsening (NIHSS $\geq$4) due to acute ischemic event during the hospital stay; 2) report of stroke on adverse events logs during the 90 day follow up (i.e. h/o neurological deficit that persisted for $\geq$ 24 hrs with NIHSS increase $\geq$1 or CT or MRI evidence of new area of infarction; and 3) any positive response to questions on the Questionnaire for Verifying Stroke Free State (QVSFS) performed at the 90day

assessment. If, based on the initial criteria for the determination of recurrent stroke, a new stroke does occur, additional information will be collected and an Adverse Events worksheet (SHINE Case Report Form) will be completed.

D.8. Discontinuation of Participation

Participation in the I-SPOT trial is voluntary and the participant may withdraw at any time. Participants who withdraw from the I-SPOT trial may continue to participate in the SHINE trial.

If all attempts fail to locate the participant fail, the participant will be considered lost to follow-up.

Participants who are enrolled in the I-SPOT study and before the 48 hour blood draw have mechanical thrombectomy performed, intra-arterial thrombolytics or systemic anticoagulants administered will still have blood drawn for the 48 hour I-SPOT laboratory studies.

D.9. Data Management

The Statistical and Data Management Center (SDMC) for SHINE is located at the Medical University of South Carolina (MUSC) in Charleston, SC and will also serve I-SPOT. MUSC serves as the SDMC for the NETT Clinical Coordinating Center and provides highest quality data coordination for multiple multicenter clinical research studies. SDMC data coordination activities include web-based Data Coordinating Unit (WebDCU™) development for data and project management, central registration and randomization, data management and archiving. Following analysis of the serum samples, the data will be entered into the WebDCU™ web-based system developed and validated at the SDMC for the SHINE trial.

The database will have extensive consistency checks programmed into the forms (eg, data type, range and logic checks). Additional consistency checks will periodically be run after data entry occurs at the laboratory. All data items that fail the programmed consistency checks will be queried via the data clarification request (DCR) process initiated by the SDMC data managers. Data obtained from I-SPOT will not be given to study sites or study investigators during the course of the study. All biomarker data will be entered directly into the WebDCU™ SHINE/I-SPOT study database by personnel in Dr. Rao's central laboratory.

At Temple University, the I-SPOT Project Manager will oversee all activities related to tracking both the blood samples and the lab results including 1) assuring sites always have an adequate number of kits for blood samples, 2) tracking blood collection at study time points, and 3) with NETT Coordinating Center Education Coordinators, provide education at the I-SPOT study initiation and periodically throughout the study to ensure compliance with methods for collection to shipping of samples. At Temple, the I-SPOT Project Manager, using the specimen tracking mechanism provided by the SDMC, will

monitor the 1) inventory of blood samples received by the Temple Research lab, 2) delivery of blood samples to the research lab, 3) track monthly data entry, and 4) work closely with the laboratory personnel and SDMC data managers

D.10. Sample Size and Statistical Analysis

The statistical analyses of the three I-SPOT aims essentially corresponds to comparing contrasts of the blood coagulation marker levels in four possible subgroups of SHINE study. The subgroups are, i) patients receiving strict hyperglycemia control (treatment) having favorable outcome (as defined in the SHINE study), ii) patients receiving strict hyperglycemia control (treatment) having unfavorable outcome, iii) patients in the standard treatment group having favorable outcome and iv) patients in the standard treatment group having unfavorable outcome. **The main dependent variable for this analysis will be the change from baseline to 48 hours in the biomarker levels.** These data from the biomarkers will be analyzed using a single two-way ANOVA in which the dependent variable is the blood coagulation marker level and the independent factors are the treatment group (namely strict hyperglycemia control and standard treatment of hyperglycemia), the response status (favorable or not as defined by the SHINE study) and the interaction between the two. The Aim 1 compares the biomarker levels of treated vs. standard, the Aim 2, hypothesis 1) compares the biomarker levels of patients with favorable vs those with not favorable outcomes (where favorable is as defined by the SHINE study) and Aim 2 hypothesis 2) compares the difference between the biomarker levels of favorable vs. not favorable patients in the treatment and standard groups (the interaction effect). The basic statistical model therefore would be, $y_{ijk} = \mu + \tau_i + \gamma_j + (\tau\gamma)_{ij} + \varepsilon_{ijk}$, where y_{ijk} represents the change in the biomarker level from baseline to 48hours of the *k*th patient in the *i*th treatment (*i* = 1 or 2; 1 = strict hyperglycemia control; 2= standard treatment) and *j*th response type (*j* = 1 or 2; 1 = responded favorably; 2 = did not respond favorably) and the ε_{ijk} represents the corresponding residual. Here, *m* represents the overall mean, *t* represents the effect of treatment, *g* represents the effect of SHINE outcome (favorable or not) and $\tau\gamma$ represents the interaction effect. Testing the null hypotheses, $H_0 : \tau_1 = \tau_2 = 0$ corresponds to Aim 1, testing $H_0 : \gamma_1 = \gamma_2 = 0$ corresponds to Aim 2, hypothesis 1, and testing $H_0 : (\tau\gamma)_{ij} = 0$ for all *i* and *j* corresponds to Aim 2, hypothesis 2.

As secondary analyses, because the blood glucose level itself may be highly variable irrespective of the treatment group, the effect of the actual blood glucose level on the outcome will be explored by including this in the model mentioned above in a covariate analysis. Given the exploratory nature of this study, secondary analyses will include demographic variables such as, age, gender, etc. and baseline measurements as covariates. Time (baseline and 48 hour) will also be explored as a fixed effect and analyze the data using a repeated measures ANOVA. Several biomarkers are being simultaneously considered, therefore correlation and scatter plot matrices will be considered. Also considered will be multiple comparisons adjustments such as the Scheffe's or Simes methods. The study will be planned mainly to establish associations between the biomarker levels and the treatment and/or SHINE outcome. A sample of 315

SHINE participants (157 from the treatment group and 157 from the control group) will be sequentially entered into the I-SPOT study. A limited number (120) of the I-SPOT subjects will have received intravenous tPA. If significant associations are observed, further randomized studies are warranted to establish causality. However, to determine the trends for planning such a study, the secondary analyses will attempt to model the biomarkers as predictors of SHINE outcome using a logistic regression model. This will be achieved by fitting the logistic regression model of the form, $logit(P(Y = 1 \mid X, T)) = \alpha + \beta X + \gamma T$, where $Y = 1$, implies the outcome was favorable (0 otherwise), X is the magnitude of decrease in the biomarker and T specifies whether the patient was treated or a control. The parameter β which represents the strength of association between the predictor X and the outcome will be tested.

Appreciating that biomarker levels may be significantly altered by IV tPA, these data will be analyzed separate from non-tPA treated patients. We will test the primary I-SPOT hypotheses and perform the planned secondary analyses including exploration of the relationship between biomarker levels and actual blood glucose level and with demographic variables such as, race, age, gender, etc. and baseline characteristics such as cardiovascular risk factors, use of VTE prophylaxis, insulin dose, etc. as covariates. Also, appreciating that the rate of change in biomarker levels may be highly variable irrespective of the treatment group, we will explore relationships between biomarker levels and independent factors and covariates at baseline and at 48 hours, with time as a fixed effect.

Sample size considerations: The study will be primarily powered to test the effect of treatment on the biomarker levels (Aim 1, hypothesis 1) and to test the differences between patients with favorable and unfavorable SHINE outcomes (Aim 2, hypothesis 1). Based on this, the expected power for testing the interaction effect will be provided using projections of number of patients in the favorable and unfavorable groups. Based on the data presented in the background section (Figure 1) and in previous studies (Vaidyula, et. al, 2006) on an average, the anticipated change from baseline to 48 hours among those receiving standard treatment (controls) is estimated to be about 40 units. Assuming the magnitude of a clinically relevant difference between treatment and control to be 25% (10 units), at an overall significance level of 5% (with a Bonferroni adjustment for 5 biomarkers leading to 1% for each biomarker), 80% power, and a s.d. of 25 units (based on the range observed in the previous studies) a total of 296 patients (148 in each treatment group) will be needed. Since the outcome status will not be known at the time of sample selection, the determination of the power for the hypotheses in Aim 2 are calculated based on projected minimum rates of favorable outcome in the SHINE study. These rates are 25% and 32% for the control and treated groups, respectively. Using these rates, anticipated favorable outcomes are 85 (37 in the control and 48 in the treatment) and 211 unfavorable outcomes. This breakdown will yield 80% power to detect a 11 unit difference. For the interaction hypothesis (Aim 2, Hypothesis 2), simulations show (using PROC GLMPOWER in SAS) this sample size will allow detections of a difference between treatment and control of 20 units in the differences among the favorable and unfavorable outcomes. An example of a detectable interaction is shown in Figure 3.

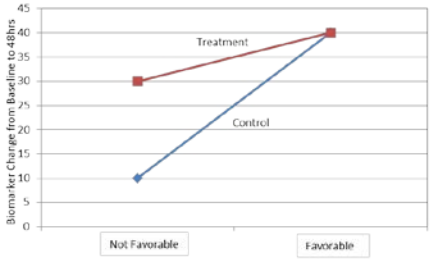


Figure 3. Example of a detectable interaction between Treatment and Control groups showing the change in biomarker levels from baseline in patients with and without favorable outcomes (as defined in the SHINE study) modulated by SHINE treatment group assignment (degree hyperglycemia control).

With respect to the logistic regression (secondary) analysis, the total sample size of 296 will allow more than 80% power to detect an odds ratio of 2.0 for one s.d. difference in the biomarker level, adjusting for the treatment effect (assuming the SHINE treatment effect size of 1.5 for a favorable outcome and at the rate of 25%). A 6% attrition rate is anticipated due to several factors. The total sample size requested is 315. Since no new treatment or intervention is part of this ancillary study, no separate interim analysis is necessary. However, in the parent SHINE study stopping rules have been defined based on interim analyses after 500, 700, 900 and 1100 patients have been entered, respectively. Therefore, potentially the study could stop earlier. Although 35% of the SHINE patients were expected to be eligible for the I-SPOT, assuming the minimum required rate of 22.5% (315/1400), and adjusting for the 6% attrition, anticipated patients at these times are 100, 139, 179 and 219. Given the same power and s.d. the detectable effect sizes for the treatment versus control comparisons would considerably increase (to 17.5, 15, 13 and 11.5 units, respectively) for these smaller sample sizes.

Since the start of I-SPOT enrollment, between 19% and 28% of SHINE enrolled patients have been eligible to participate in the I-SPOT study. The most common exclusion to I-SPOT eligibility has been treatment with IV tPA; and the percentage of patients treated acutely with tPA rose from 58% to 68% with the largest increases occurring over the past 6 months. At this rate, we project the sample enrollment size for this cohort to be limited to 195 patients. Enrollment of I-SPOT patients treated with tPA will be limited to 120, with the total ISPOT enrollment remaining at 315 subjects.

The primary analysis of non-tPA-treated ISPOT study subjects will not be compromised. We determined that, given the observed variability in measurements of samples thus far, there is sufficient power to detect even a fairly conservative effect size with a sample of 96 subjects per SHINE group of a total of 192 subjects.

Appreciating that biomarker levels may be significantly altered by IV tPA, data from IV tPA treated I-SPOT subjects (n=120) will be analyzed separate from non-tPA treated subjects. We will test the primary I-SPOT hypotheses and perform the planned secondary analyses as above.

D.10. Administrative Management

I-SPOT Organization and Relationship to SHINE: Participating sites include Hub and Spoke hospitals associated with the Neurological Emergencies Treatment Trials (NETT) as well as multiple non-NETT hospitals that are primarily academic centers with strong infrastructure to conduct clinical research on AIS. The NETT is an NIH-NINDS funded network of a Clinical Coordinating Center (CCC) at the University of Michigan, a Statistical and Data Management Center (SDMC) at the Medical University of South Carolina and 17 Hubs, which are located at large academic medical centers. Each Hub has from 2-11 satellite hospital sites for enrollment of patients in clinical trials. This network has been devised to maximize the efficiency and quality of clinical research care in acute neurological clinical trials. In addition to the NETT sites, there will be 11 non-NETT sites that will also enroll subjects into the SHINE trial. All participating sites have extensive experience enrolling patients in complex trials requiring rapid initiation in the emergency department setting.

I-SPOT Executive Committee (EC). The Executive Committee will convene by telephone every month (or more often, if necessary) to develop and operationalize study Standard Operating Procedures (SOPs) relating to protocol training, protocol adherence, review study progress and quality control data on sample testing, and discuss any obstacles in accomplishing study objectives. The committee will also assist the investigators in analysis and interpretation of the final results of the study. The EC will be comprised of the Co-PIs, I-SPOT Project manager and Lead Coordinator; a member of the SHINE executive committee (Dr. Askiel Bruno), three members of the NETT executive committee (Drs. William Barsan and Arthur Pancioli and NETT Executive Director, Valerie Stevenson), and one member of the SHINE and I-SPOT SDMC at MUSC (Dr. Viswanathan Ramakrishnan).

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