Supplementary methods:

Sera processing and analysis of metabolites:

We acquired sera from two previous NIH-funded clinical trials to probe for the uremic solute-AHR-TF axis after an Institutional Review Board approval. DAC and TIMI-II trial cohorts served as appropriate datasets, given thrombotic events in the patients undergoing vascular interventions such as fistula surgery or balloon angioplasty, respectively, and the availability of serum samples. The samples were thawed once, aliquoted in smaller volume for different assays and snap frozen immediately and were stored in a dedicated -80 freezer. The sera samples were subjected to a targeted metabolomics analysis using methods previously described[11, 19]. NHLBI deems a sample 'low impact' if the serum is available in sufficient quantity and will not get depleted to more than 50% of the current amount after sharing for a proposed study. We acquired 377 'low impact' sera samples from TIMI-II trial and 473 samples from DAC-fistula trial for this study.

Stability of the solutes and functional assays:

This study examined human sera samples from two clinical trials that were performed several years ago (DAC in 2003-2007 and TIMI-II in 1986-1988). As these samples were frozen and stored at -80^{0}C, we examined the effect of storage on the stability of the metabolites and AHR and TF activity levels. Specifically, the levels of metabolites, AHR and TF activities in the same set of sera samples were analyzed twice over four years of storage (2012-2016). Ten patients with end stage renal disease (ESRD) treated at Boston University Medical Center were selected from a group described previously[9, 11]. This group consisted of ten African American ESRD patients predominantly males (N = 8) with median age 42 years (range 31-53 years). While testing the effect of the entire storage period of

these two clinical trials was not feasible, our results showed no significant effect of four years of storage on the levels of the metabolites and AHR or TF activities (**Supplementary Figure 5A-5C**).

Cell lines:

Primary human aortic vSMCs obtained from ATCC were grown in DMEM low glucose with 5% calf serum and 1% penicillin and streptomycin and used up to 10 passages. HepG3 cells and HUVEC-tert cells were grown DMEM with high glucose and 5% calf serum and 1% penicillin and streptomycin.

For TF activity, we used both primary human aortic vascular smooth muscle cells (vSMCs) and endothelial cells. For the DAC-fistula study, TF activity was also analyzed in the immortalized human umbilical endothelial cells (HUVEC-tart). While we considered the option of using primary human umbilical endothelial cells (Lonza, USA), we observed a batch-to-batch variation in response to TF activity. For example, a set of sera samples elicited TF activity in three independent batches of HUVECs with coefficient of variation (CV) by 38.5%, suggesting their unsuitability to be used for such study. Therefore, we immortalized HUVCs and after performing both a biochemical characterizing of the cell line using endothelial cell-specific markers and functional characterization with an in vitro angiogenesis assay for up to 15 passages, we used for TF activity assay. This provided us with sufficient number of passages to allow screening of 870 samples with minimal variability (CV 13-18%) of TF activity over 6-8 passages). These immortalized HUVCs called HUVEC-tert cells, which were generated by transducing the primary pooled human umbilical vein endothelial cells (HUVECs, Lonza, USA) with a retrovirus expressing telomerase.

The persistence of endothelial characteristics over 15 passages was confirmed by different ways as described below. The lysates from these cells were probed for vascular endothelial cadherin (VE cadherin), which still persisted in HUVEC-tert similar to HUVECs, but not in NIH 3T3 fibroblast cells and HK-2 cells (human kidney epithelial cells) (**Supplementary Figure 6A**). Functional evaluation of these cells was performed using *in-vitro* angiogenesis or tube formation assay (**Supplementary Figures 6B and 6C**)**.** 15,000 HUVEC-tert cells were seeded in 96-well plates precoated with 100 µl of growth factor-poor Matrigel™ (BD Biosciences) (10 mg/ml) and incubated at 37°C for 24 hours. Tube formation was imaged using phase contrast microscopy and tube lengths were analyzed using ImageJ software. The tube formation with HUVEC and HUVEC-tert samples was similar. No tube formation was observed with fibroblast or epithelial cells. These data confirm the persistence of the endothelial phenotype in HUVEC-tert cells up to 15 passages. TF activity was examined within 10 passages of these cells.

AHR activity:

AHR Activity was measured in HUEC-tert, as described previously (**Supplementary Figure 1A**) [11]. Briefly, the cell lines stably expressing a Cignal Lenti Reporter xenobiotic response element tethered to luciferase reporter (XRE-luc) (Qiagen, CLS-9045L-8) were used. The cells seeded at 1000/well in 96-well plate were serum starved for 16 hours and then treated with the serum with or without AHR inhibitor, CH223191 for 24 hours. Firefly luciferase activity was measured using luciferase assay kit (Promega# E1501). After luciferase assay, the cells were lyzed in RIPA buffer and the protein content was

determined using Bradford assay. The luciferase signal was then normalized to protein content (relative luciferase activity unit).

TF procoagulant activity:

TF surface or surface procoagulant activity was measured using a two-step FXa generation assay, as described previously[11] (**Supplementary Figure 1B**). A standard curve was generated by incubating human recombinant lipidated TF (Enzo lifesciences, Cat# SE-537) ranging from 0-500 pM along with 5 nM of human factor VIIa (Enzyme Research Laboratories Cat# HFVIIa) and 150 nM of factor X (Enzyme Research Laboratories Cat# HFX 1010) and $CaCl_2$- 5 mM for 30 minutes at 37^0C. The reaction mixture was incubated with chromogenic substrate for factor Xa (Chromogenix Cat# S2765, 1mM-final concentration). The reaction was stopped after 5 minutes using 10 ul of 50% glacial acetic acid and read at 405 nm absorbance.

In vSMCs, the TF procoagulant activity was examined in a 96 well plate format with 1000 vSMCs seeded per well. The cells were serum starved for 16 hours and treated with 1% sera for 24 hours. Cells were washed with Tris Buffer Saline (TBS) (50 mM Tris HCl, 120 mM NaCl, 2.7 mM KCl, 3mg/ml BSA, pH=7.4) and incubated with 55 ul TBS containing 5 nM of human factor VIIa and 150 nM of factor X and $CaCl_2$- 5mM for 30 minutes at 37^0C. 50 ul of the supernatant was incubated with chromogenic substrate at 1 mM-final concentration. The reaction was stopped after 5 minutes by adding 10 ul of 50% glacial acetic acid and the absorbance was read at 405 nm. TF activity levels in samples were calculated using regression analysis. To confirm the specificity of the TF activity assay, activity was measured in cells pre-treated with anti-TF neutralizing antibody (50 ug/ml)

(1H10) or control antibody for an hour, as described previously[11]. TF activity was normalized to the number of cells.

The number of viable cells was determined using Alamar Blue assay (Thermofisher Scientific). To create the standard curve, serial dilution of VSMCs cells was performed in a black, clear-bottom 96 well plate. Cells were then treated with Alamar Blue for one hour. After one hour, the fluorescence intensity of Alamar Blue was measured at 570 nm using Infinite microplate reader from Tecan. The fluorescence readout using Alamar Blue is linear over a range of ~500 to 50,000 cells per manufacturer insert. By serial dilution of cells, we obtained a linear standard curve with $R^2 = 0.97$ (**Supplementary Figure 6D)**. For normalization of TF activity, after TF activity measurement, the cells were washed with 1X PBS and 100 µl of media was added and incubated with Alamar Blue for one hour at 37 ºC in CO_2 incubator. The fluorescence was measured at 750 nm and standard curve was applied to obtain the number of viable cells.

Determining the optimum concentrations of the sera used for the DAC and TIMI-II studies:

Before examining the samples for AHR and TF activity assays, we determined the optimal concentration of serum for the DAC and TIMI-II trials to be used for these assays. A titrated concentration of sera from five randomly selected subjects from the DAC trial was used on HepG2 and vSMCs for AHR and TF activities, respectively. The specificity of both AHR and TF activation was further confirmed by co-treating the sample with a competitive AHR inhibitor – CH223191[30], which has been previously shown to inhibit both the AHR and TF activities[11]. Our results showed that one percent and five percent of sera resulted in the highest level of AHR and TF activities, respectively, and were suppressed by 70-80% with

CH223191 (**Supplementary Figures 7A-7B**). A similar analysis was performed in TIMI-II samples (**Supplementary Figures 7C and 7D)**. One percent of serum resulted in the highest level of both AHR and TF activities, respectively, which were suppressed with CH223191. Based on these results, for DAC study, we used 1% serum for AHR Activity and 5% serum for TF activity assay, while 1% serum was used for both the assays in TIMI-II study.

Cell lysis and Western blotting:

Cell harvest and immunoblotting have been described previously[9]. Monoclonal antibodies specific for tissue factor (Thermoscientific), GAPDH (Cell signaling) 1:1000 dilutions were used. CH223191 was purchased from Sigma (Catalogue C8124) and was dissolved in DMSO.

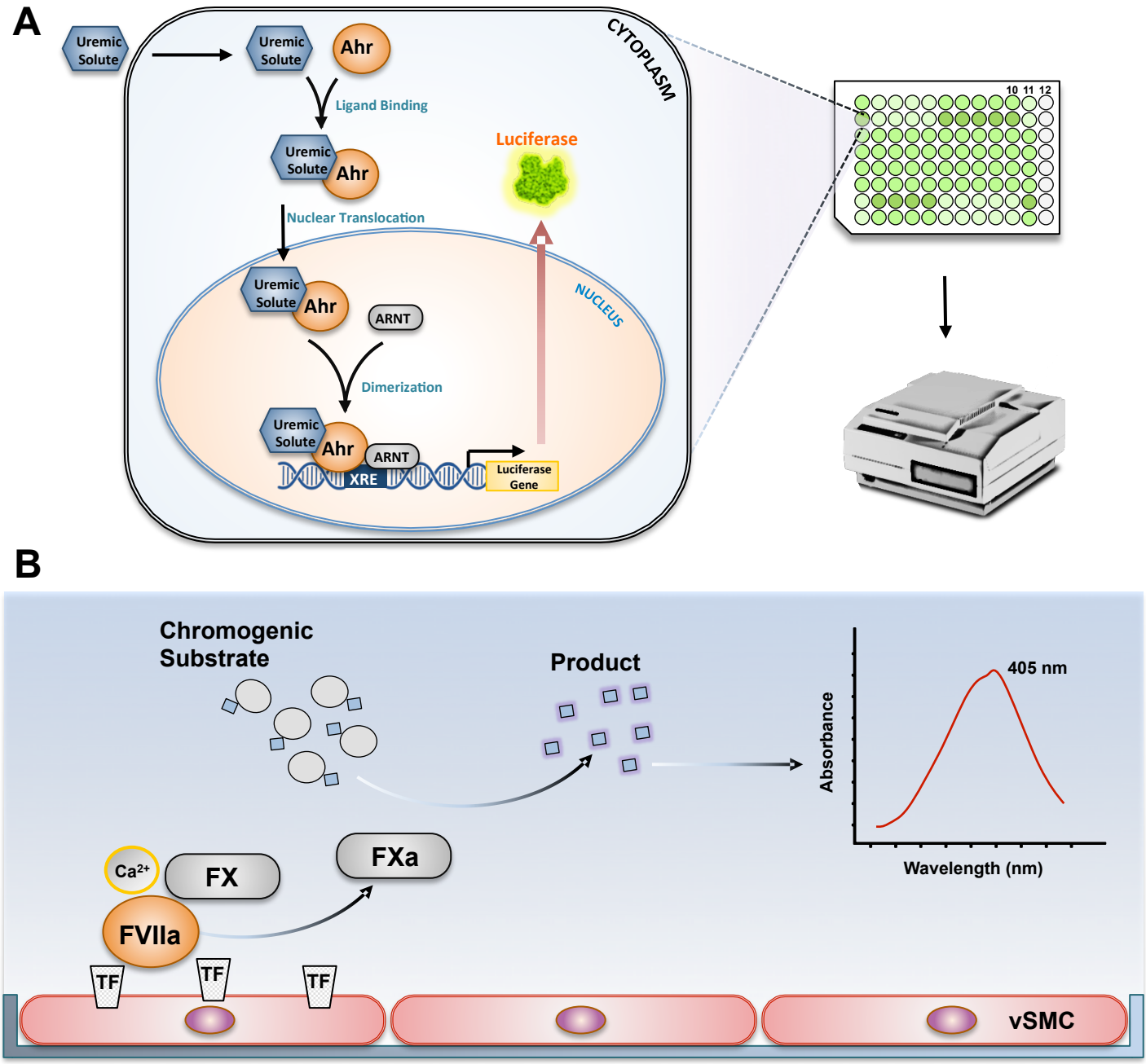
Institutional approval:

All experiments were performed under IRB protocol H-32525, reviewed and approved by Boston University. Animal studies were approved by IACUC protocol # A.N15449.

Statistical analysis:

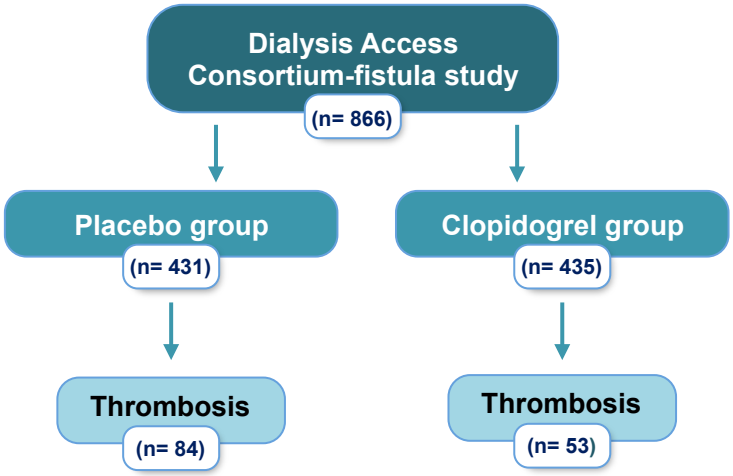
Summary statistics are presented as the mean, median and SD. Either a Student's t-test or Fisher's exact test was performed to compare the groups as appropriate. Spearman or Pearson correlation was performed to analyze correlation between two variables, as appropriate. Statistical significance was assessed at $p<0.05$.

Supplementary Figure 1

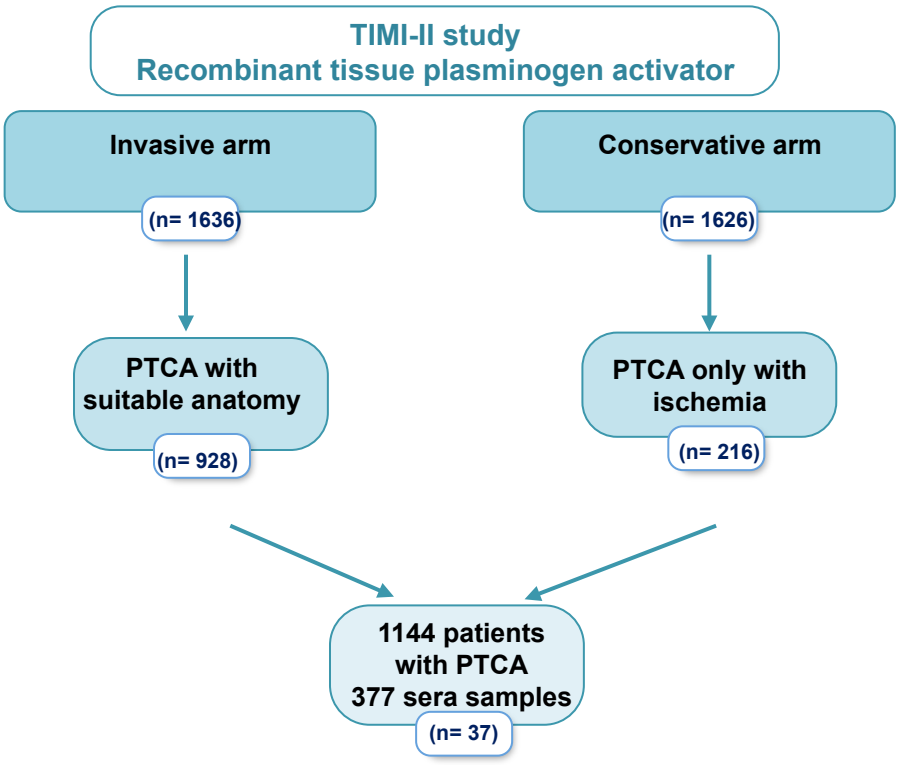


Supplementary Figure 1

C



D



Supplementary Figure 1. Trial design

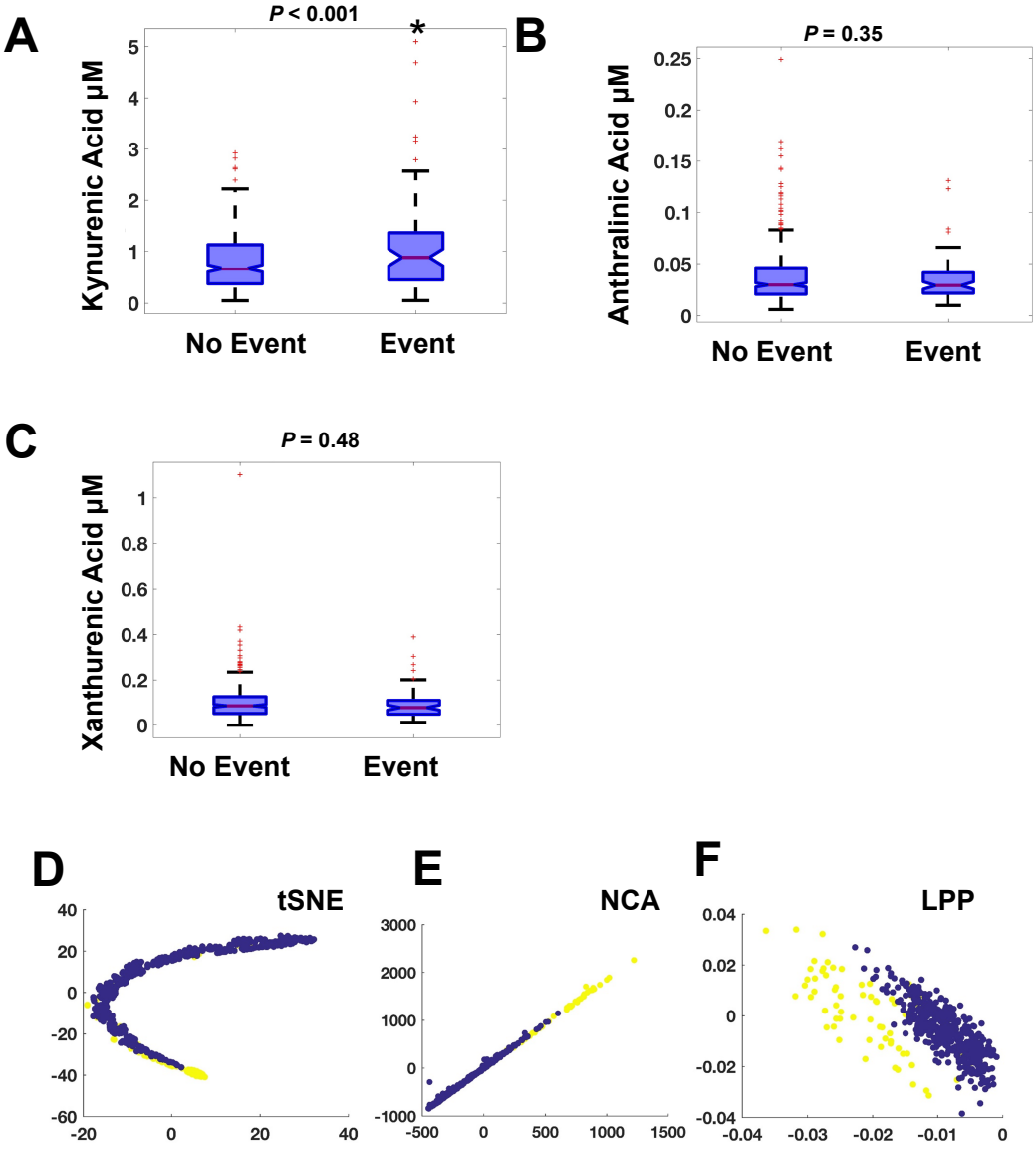
(**A**). **AHR activity assay**: AHR activity was examined in HUVEC-tert cells stably expressing Cignal Lenti Reporter xenobiotic response element tethered to luciferase reporter gene (XRE-luc). Cells seeded at 1000/well in a 96-well plate were serum starved for 16 hours and treated with a pre-defined serum concentration. The firefly luciferase measured using luciferase assay was normalized to protein concentration.

(**B**). **TF activity assay**: TF surface/procoagulant activity was measured using a two-step FXa generation assay in a 96 well plate format seeded with primary human aortic vascular smooth muscle cells (vSMCs) or human umbilical vein endothelial cells. The cells were treated with pre-defined serum concentration, followed by incubation with human factor VIIa and factor X and $CaCl_2$. The TF converts Factor X to Xa, which is measured using a chromogenic substrate that changes color measured at 405 nm. A standard curve generated using recombinant TF provides the amount of TF activity and normalized to 10^3 cells.

(C). DAC was a randomized, double-blind, placebo-controlled trial conducted from 2003-2007 with 877 participants with end stage renal disease (ESRD) or advanced chronic kidney disease who underwent surgical creation of an arteriovenous fistula for hemodialysis. Participants were randomly assigned to receive clopidogrel (n = 441) or placebo (n = 436) for 6 weeks starting within 1 day after fistula creation. The primary outcome was fistula thrombosis, determined by physical examination at 6 weeks. The secondary outcome was failure of the fistula to become suitable for dialysis defined as use of the fistula at a dialysis machine blood pump rate of 300 mL/min or more during 8 of 12 dialysis sessions during the 5^{th} month after fistula creation. Fistula thrombosis occurred in

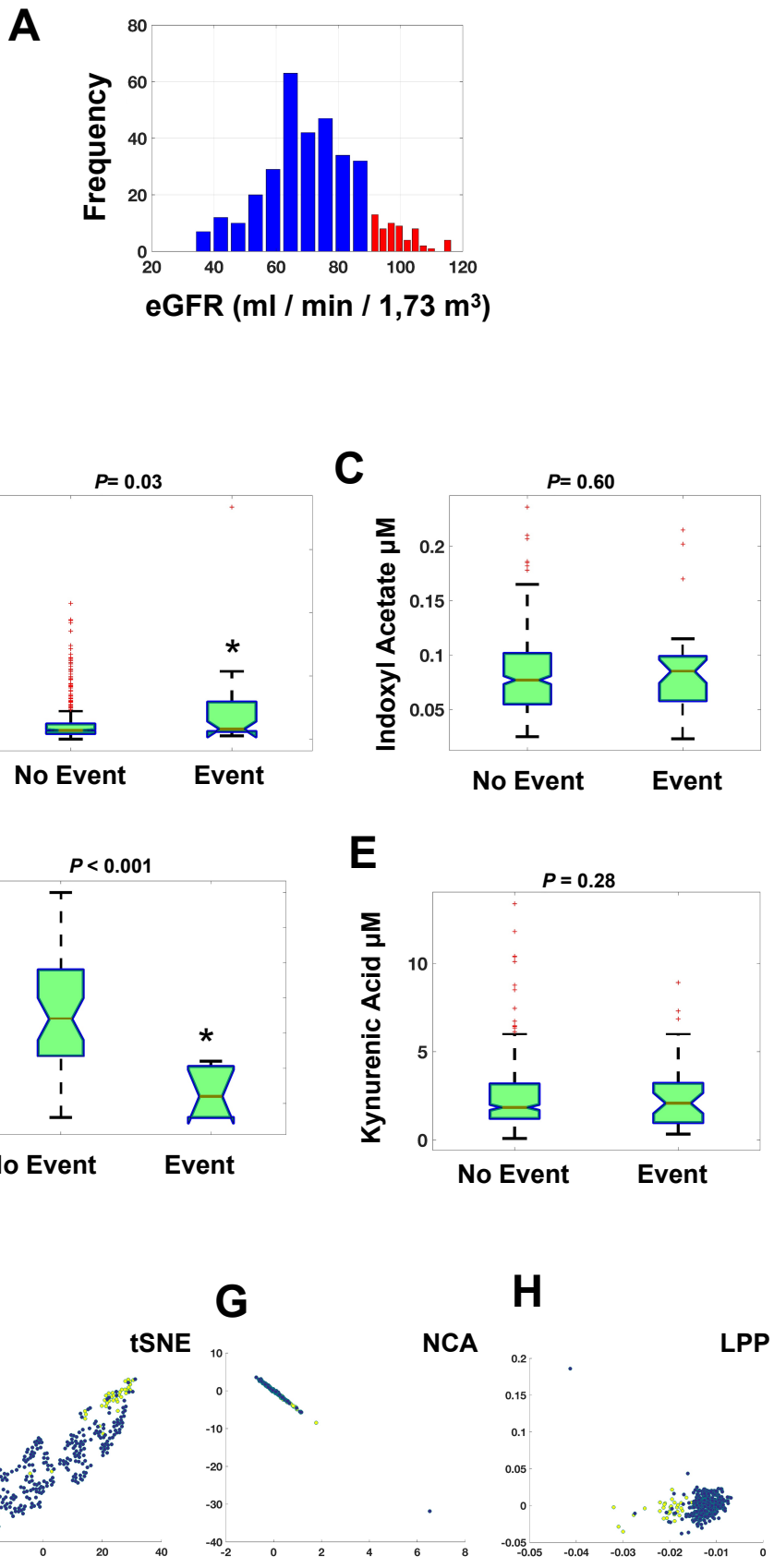
53 (12.2%) participants assigned to clopidogrel compared with 84 (19.5%) participants assigned to placebo. Failure to attain suitability for dialysis did not differ between the clopidogrel and placebo groups (61.8% vs 59.5%, respectively).

(D). TIMI-II was a randomized interventional multicenter trial conducted from 1993-1998 with 3262 participants who were treated with intravenous recombinant plasminogen activator within four hours of onset of chest pain with ST elevation myocardial infarction. Of the total participants, 1636 were randomized to receive coronary angiography within 18-48 hours of treatment of recombinant plasminogen activator followed by prophylactic percutaneous transluminal coronary angioplasty (PTCA) if arteriography demonstrated suitable anatomy (invasive arm). 1626 participants were randomized to a conservative strategy wherein patients underwent arteriography and PTCA only with spontaneous or exercise-induced ischemia. In the invasive arm, PTCA was attempted in 928 of 1636 patients. In the conservative arm, 216 patients underwent PTCA. Serum samples from 377 of 1144 patients who underwent PTCA were tested. Among the 377 participants with the baseline serum samples, 37 patients had reinfarction or reocclusion after PTCA, which were considered as ‘events’ for the current study.


A
P < 0.001
Kynurenic Acid µM
No Event
Event
B
P = 0.35
Anthralinic Acid µM
No Event
Event
C
P = 0.48
Xanthurenic Acid µM
No Event
Event
D
tSNE
E
NCA
F
LPP

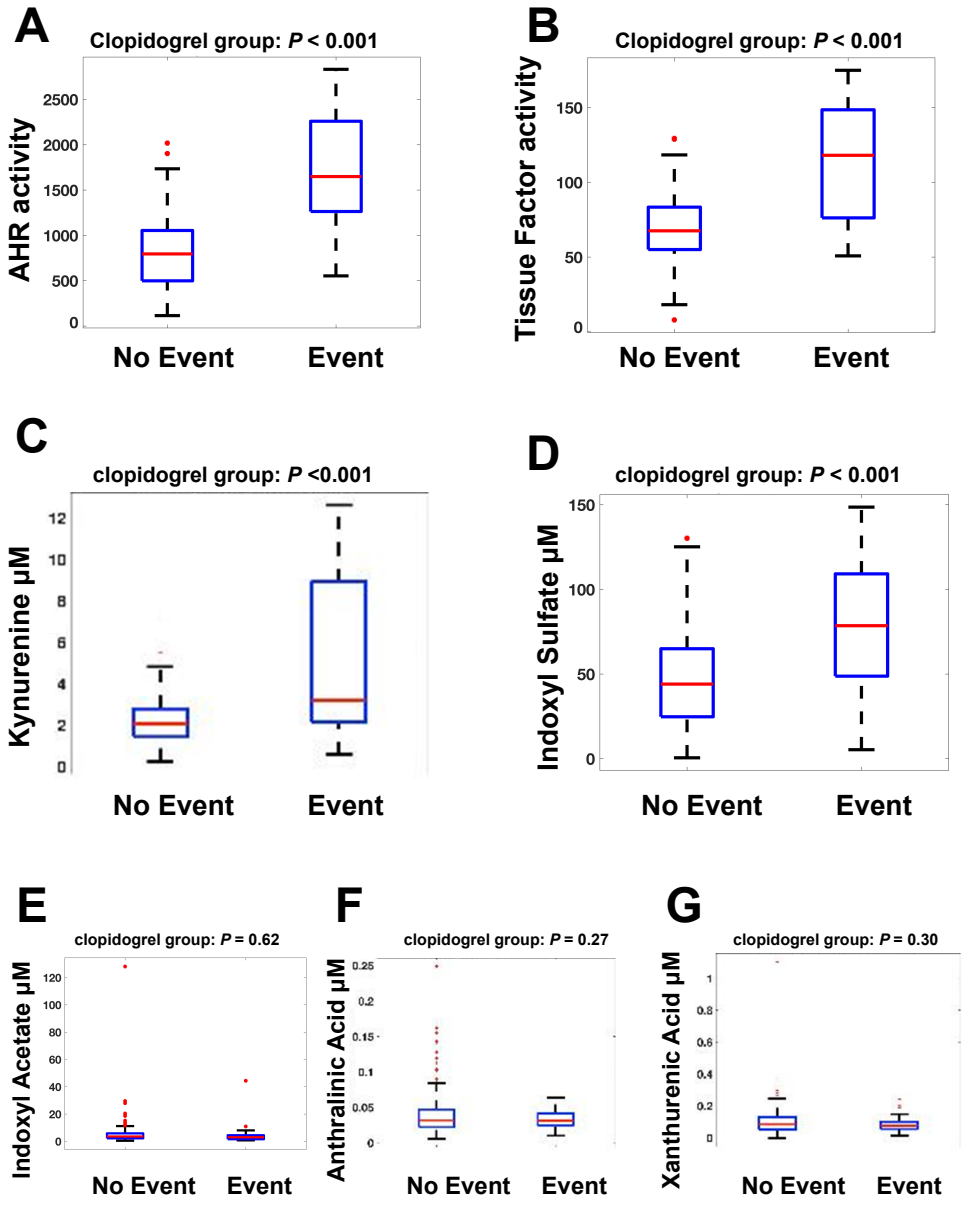
Supplementary Figure 2. Metabolites and pattern recognition algorithms in DAC-fistua trial

(A, B, C) Box plots showing the differences in the metabolites between the AVF thrombosis 'event' and 'no-event' groups. **(D, E, F)** Different dimensionality reduction techniques such as t-distributed stochastic neighbor embedding (t-SNE), neighborhood component analysis (NCA) and locality preserving projections (LPP), respectively were used to transform the metabolite-activity data into two dimensions, and represented in plots. The patients belonging to the AVF thrombosis 'event' group are shown in yellow and ones in the 'no-event' group are shown in blue.

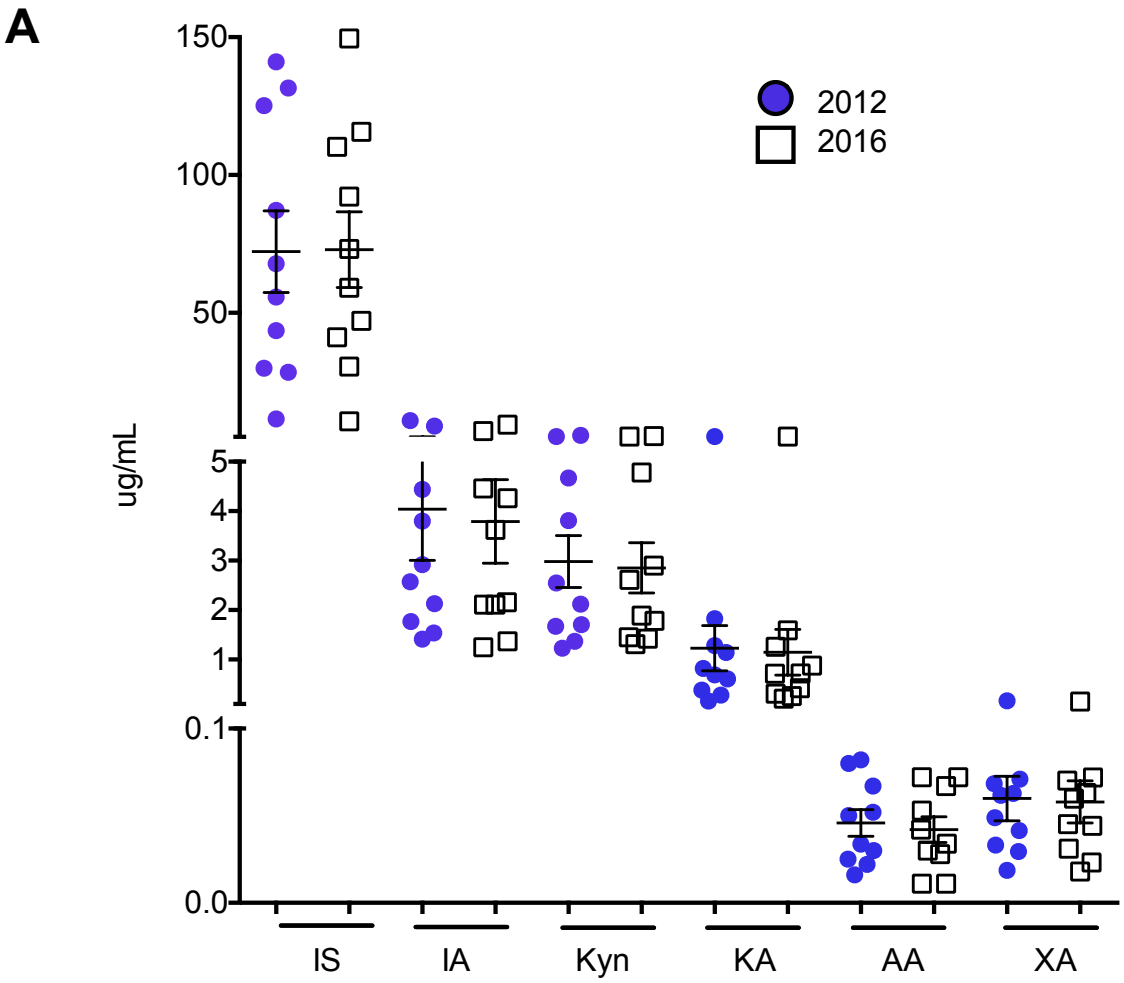

A
Frequency
eGFR (ml / min / 1,73 m³)
B
P= 0.03
Xanthurenic Acid µM
No Event
Event
C
P= 0.60
Indoxyl Acetate µM
No Event
Event
D
P < 0.001
Anthralinic Acid µM
No Event
Event
E
P = 0.28
Kynurenic Acid µM
No Event
Event
F
tSNE
G
NCA
H
LPP

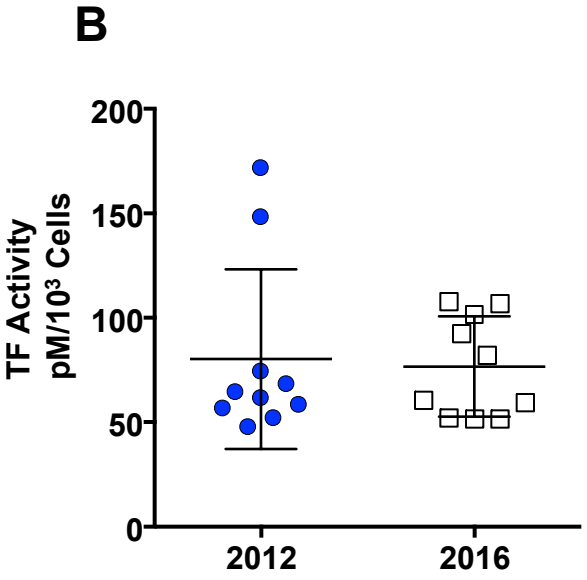
Supplementary Figure 3. Metabolites and pattern recognition algorithms in TIMI-II trial

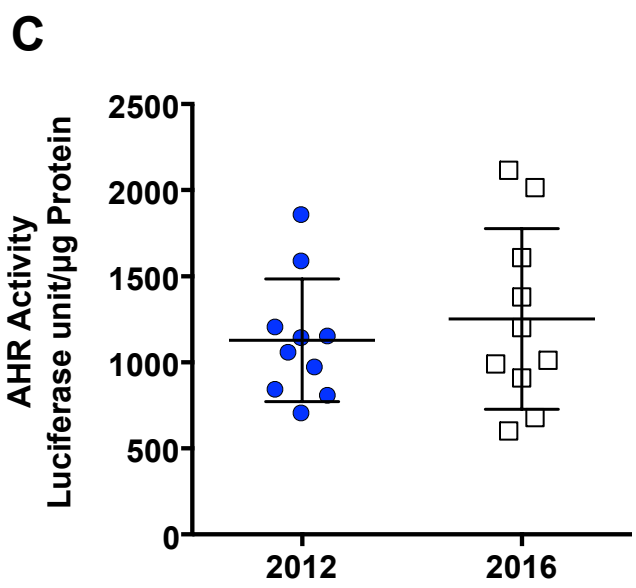
(A) Histogram representing the eGFR distribution within the TIMI-II patient cohort. The eGFR of > 90 ml/min calculated using CKD-EPI equation was considered normal (shown in red). CKD was defined as patients with eGFR < 90 ml/min (shown in blue). **(B, C, D, E)** Box plots showing the differences of different metabolites between the 'event' and 'no-event' groups, as defined in the text for the TIMI-II trial. **(F, G, H)** Different dimensionality reduction techniques such as t-SNE, NCA, and LPP, respectively, were used to reduce the data into two dimensions, and show in a plot. The patients belonging to the 'event' group are shown in yellow and ones in the 'no-event' group are shown in blue.


A
Clopidogrel group: P < 0.001
AHR activity
2500
2000
1500
1000
500
0
No Event
Event
B
Clopidogrel group: P < 0.001
Tissue Factor activity
150
100
50
0
No Event
Event
C
clopidogrel group: P <0.001
Kynurenine µM
12
10
8
6
4
2
0
No Event
Event
D
clopidogrel group: P < 0.001
Indoxyl Sulfate µM
150
100
50
0
No Event
Event
E
clopidogrel group: P = 0.62
Indoxyl Acetate µM
120
100
80
60
40
20
0
No Event
Event
F
clopidogrel group: P = 0.27
Anthralinic Acid µM
0.25
0.2
0.15
0.1
0.05
0
No Event
Event
G
clopidogrel group: P = 0.30
Xanthurenic Acid µM
1
0.8
0.6
0.4
0.2
0
No Event
Event

Supplementary Figure 4. Activation of the thrombosis axis in the clopidogrel group. A subgroup analysis of patients who received clopidogrel in the DAC fistula study is shown. Box plots show the differences between the AVF thrombosis 'event' and 'no-event' groups for the TF and AHR activities (A and B, respectively) and individual metabolites (C-G).


A
150
100
50
5
4
3
2
1
0.1
0.0
ug/mL
2012
2016
IS
IA
Kyn
KA
AA
XA


B
200
150
100
50
0
TF Activity
pM/10³ Cells
2012
2016


C
2500
2000
1500
1000
500
0
AHR Activity
Luciferase unit/µg Protein
2012
2016

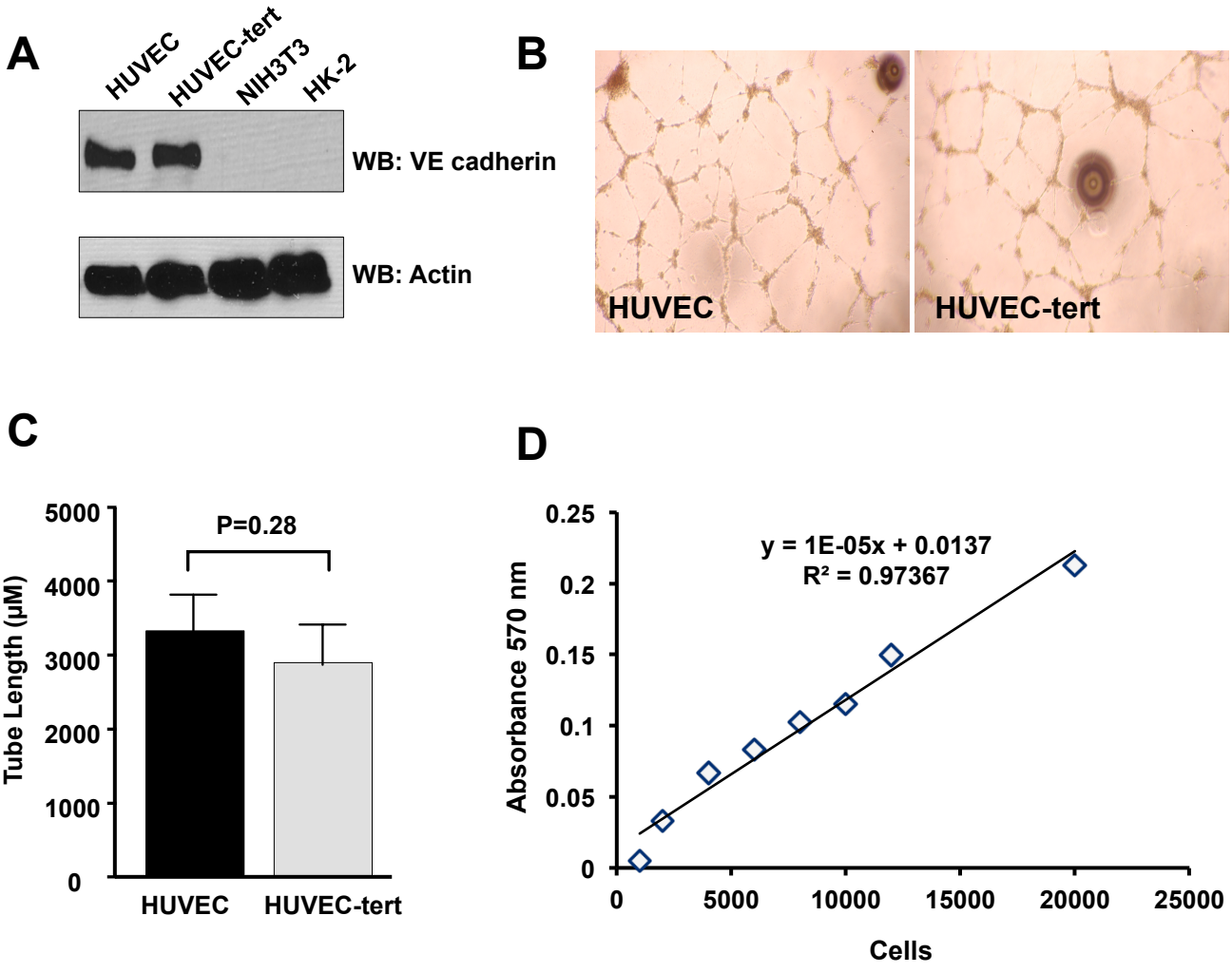
Supplementary Figure 5. Pre-analytic variability and the effect of storage of the components of thrombosis axis

(A) No effect of storage on the stability of metabolites. A set of ten sera samples from patients with end stage renal disease (ESRD) treated at Boston Medical Center was selected from a previously described group of 20 patients[9] to test the effects of storage on stability of indoxyl sulfate (IS), indoxyl acetate (IA), kynurenine (Kyn), kynurenic acid (KA), and anthranilic acid (AA) and xanthurenic acid (XA). Serum samples frozen and stored at -80°C in two separate aliquots were examined in 2012 and 2016. There were no significant differences observed in the concentration of these uremic solutes in the samples from 2012 when compared to the same samples analyzed in 2016 (non-parametric *t*-test, $p<0.05$). The specific p-values for each metabolite using a two-tailed *t*-test were 0.857 (IS), 0.459 (IA), 0.392 (Kyn), 0.403 (KA), and 0.704 (AA) and 0.362 (XA).

(B) No significant effect of four years of storage on TF Activity assay. TF activity was performed on ten samples randomly selected from a set of twenty ESRD patients as described above. The surface procoagulant TF assay was performed, as described previously on vSMCs[11] on the same set of sera stored in aliquots over four. There was no significant difference in TF activity in the samples from 2012 when compared to the same samples analyzed in 2016 (non-paramedic *t*-test, $p<0.05$). The specific p-value using a Student's t-test (two tailed) was 0.726.

(C) AHR activity did not differ in samples stored over four years period. AHR activity was performed in the same aliquots of sera stored over four year period, as described previously[11]. There was no significant difference in AHR activity in the samples from 2012

when compared to the same samples analyzed in 2016 (non-paramedic *t*-test, $p<0.05$). The specific p-value using a two-tailed *t*-test was 0.273.


A
HUVEC
HUVEC-tert
NIH3T3
HK-2
WB: VE cadherin
WB: Actin
B
HUVEC
HUVEC-tert
C
P=0.28
Tube Length (μM)
5000
4000
3000
2000
1000
0
HUVEC
HUVEC-tert
D
y = 1E-05x + 0.0137
R² = 0.97367
Absorbance 570 nm
0.25
0.2
0.15
0.1
0.05
0
0
5000
10000
15000
20000
25000
Cells

Supplementary Figure 6. Persistent endothelial features in HUVEC-tert cells after 15 passages.

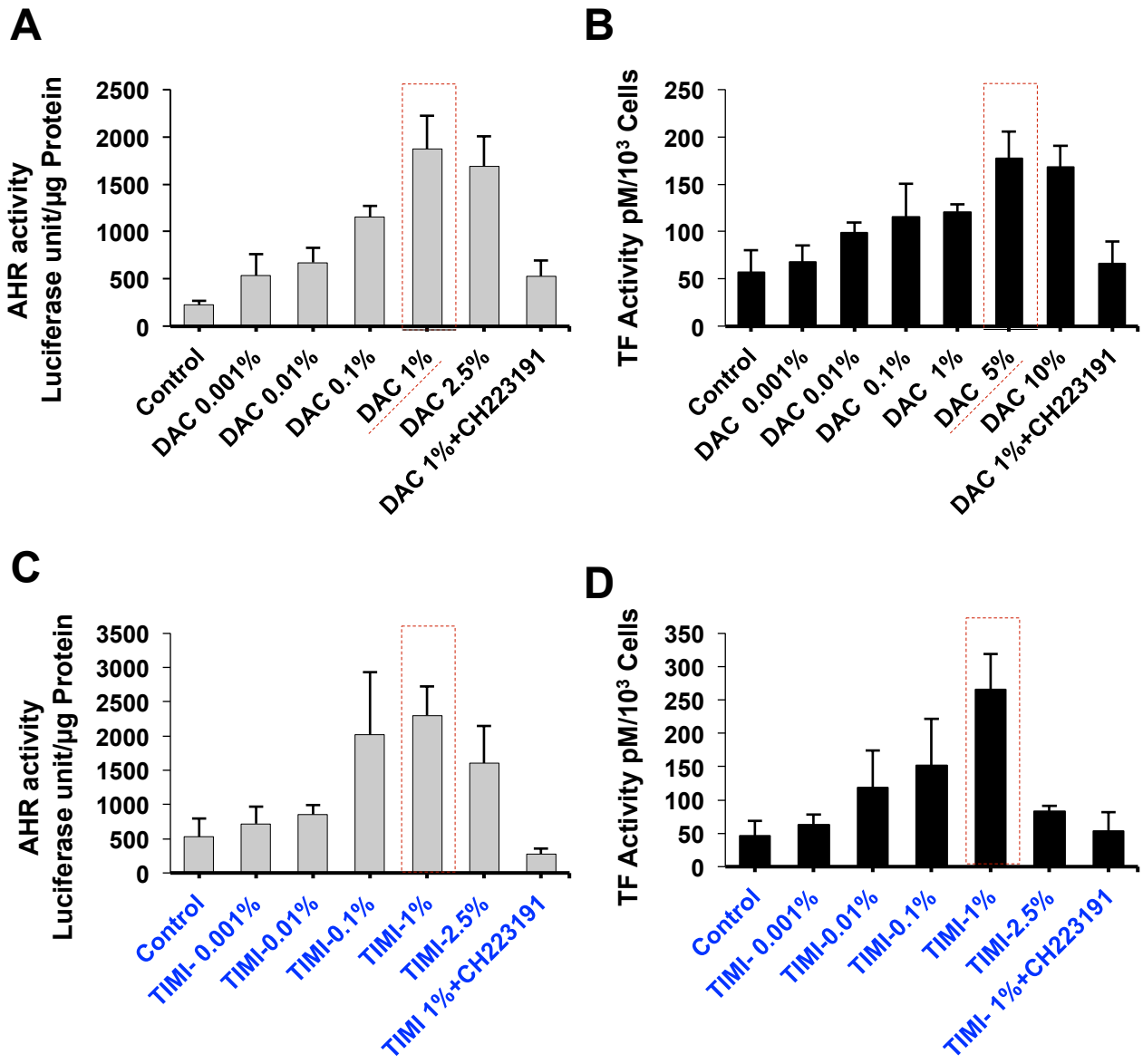
(A). Immunoblotting of the endothelial marker VE cadherin in HUVEC (4 passages), HUVEC-tert (15 passages). Note the absence of expression in negative controls NIH3T3 (mouse embryo fibroblastic cell line), and HK-2 (human kidney cortex/proximal tubule epithelial cell line). The lysates were probed for VE-cadherin (an endothelial marker) and Actin served as a control.

(B). *In vitro* angiogenesis assay of HUVEC and HUVEC-tert cells. Cells were seeded in a growth factor poor Matrigel™-coated 96 well plate and analyzed for tube formation after 24 hours of culture. Representative image of three independent experiments is shown. The tube formation is a biological assay, which supports the retention of endothelial feature of HUVEC-tert cells.

(C). Tube lengths were quantified using Image J. Mean of two separate experiments performed in triplicate is shown. Six images were evaluated in each well. Student's t-test with Bonferroni's correction was performed to determine statistical significance. Error bars = SEM. The significant difference in the tube length was observed between these cells.

(D). Standard curve of vSMC numbers in MTT assay. Serial dilutions of vSMCs counted using a cell counter were seeded in in 6 wells of 96-well plate done in two duplicate sets. One set was subjected to MTT assay using Alamar Blue® after 24 hours of seeding and other set was trypsinized and counted using a cell counter. The average of number of cells in 6 wells (X-axis) was plotted against the average absorbance values of Alamar Blue at 570nm (Y-axis). Resulting regression equation (inset) was used to determine the number

of cells in subsequent MTT assays. The number of cells was used to normalize the surface procoagulant TF activity assay.


A
AHR activity
Luciferase unit/µg Protein
2500
2000
1500
1000
500
0
Control
DAC 0.001%
DAC 0.01%
DAC 0.1%
DAC 1%
DAC 2.5%
DAC 1%+CH223191
B
TF Activity pM/10³ Cells
250
200
150
100
50
0
Control
DAC 0.001%
DAC 0.01%
DAC 0.1%
DAC 1%
DAC 5%
DAC 10%
DAC 1%+CH223191
C
AHR activity
Luciferase unit/µg Protein
3500
3000
2500
2000
1500
1000
500
0
Control
TIMI- 0.001%
TIMI-0.01%
TIMI-0.1%
TIMI-1%
TIMI-2.5%
TIMI 1%+CH223191
D
TF Activity pM/10³ Cells
350
300
250
200
150
100
50
0
Control
TIMI- 0.001%
TIMI-0.01%
TIMI-0.1%
TIMI-1%
TIMI-2.5%
TIMI- 1%+CH223191

Supplementary Figure 7. Determining the optimum serum concentration for the DAC and TIMI-II samples.

A set of five randomly selected subjects from DAC (panels **A and B**) and TIMI-II (panels **C and D**) were used to titrate the optimal concentration of serum for maximal signal detection in AHR (panels A and C) and TF (panels B and D) activity assays. Control: serum from healthy subjects. CH223191: AHR inhibitor. The activity assays were performed in three independent experiments each done in duplicate. An average of reading of AHR and TF activities represented in luciferase unit/μg protein and TF activity $pM/10^3$ cells, respectively, are shown. The values in box indicate the serum concentration used for both the assays in these two trials.