

# Thoracic Aortic Dissection in Tetralogy of Fallot: A Review of the National Inpatient Sample Database

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**Background**—Thoracic aortic aneurysm is common in patients with tetralogy of Fallot (TOF); the incidence of thoracic aortic dissection (TAD) is unknown, but generally considered to be uncommon. The purpose of this study was to determine incidence and risk factors for TAD in TOF patients.

**Methods and Results**—This work is a retrospective review of the National Inpatient Sample (NIS) database for cases of ascending TAD among all hospital admissions in adults with TOF, 2000–2014. Of 18 353 admissions in TOF patients, 11 (0.06%; 6 per 10 000 admissions) of these were TAD-related admissions. For the TAD-related admissions, mean age was  $49.8 \pm 7.2$  years; aortic surgical interventions were performed during 8 of the admissions, and overall in-hospital mortality was 45% (5 of 11). Risk factors associated with TAD-related admission were age  $>60$  years (odds ratio, 2.41; 95% CI, 1.23–3.25;  $P=0.013$ ), male sex (odds ratio, 6.91; 95% CI, 4.85–8.54;  $P<0.001$ ), and hypertension (odds ratio, 1.74; 95% CI, 1.06–3.19;  $P=0.037$ ).

**Conclusions**—This is the first population-based study of TAD outcomes in patients with TOF, and it showed a low risk of TAD in this population. This has important clinical implication with regard to surveillance and surgical intervention. Given that prevalence of thoracic aortic aneurysm is higher in TOF patients compared with the general population; therefore, making clinical decisions based on outcomes data and practice guidelines derived from patients with degenerative and bicuspid aortic valve-related aortopathies may lead to *overtreatment*. Further studies are required to better understand the pathobiology of this disease in order to make evidence-based recommendations for surveillance and treatment. (*J Am Heart Assoc.* 2019;8:e011943. DOI: 10.1161/JAHA.119.011943.)

**Key Words:** aortic aneurysm • tetralogy of Fallot • thoracic aortic dissection

Thoracic aortic aneurysm is a common complication in patients with tetralogy of Fallot (TOF), and previous studies have proposed potential risk factors for aortic aneurysm in this population.<sup>1–4</sup> Some of the risk factors include male sex, right aortic arch, history of palliative shunt, and underlying diagnosis of TOF with pulmonary atresia.<sup>1–4</sup> Thoracic aortic dissection (TAD) is a devastating complication that can occur in patients with aneurysms, and the goal of imaging surveillance is to identify patients who are considered high risk for TAD and identify possible indications for surgical intervention.<sup>5–7</sup>

Although several studies have shown a high prevalence of thoracic aortic aneurysm in the TOF population, none of these studies have reported any cases of TAD and, as a result, TAD is generally considered uncommon after TOF repair.<sup>8–10</sup> There are no population-based studies of TAD incidence and outcomes, and the available data about TAD in TOF patients are derived from case reports.<sup>11–13</sup> In the absence of epidemiological data about the risk of TAD in this population, decisions regarding surgical interventions are based on extrapolations from natural history studies conducted in patients with syndromic, degenerative, and bicuspid aortic-valve-related aortopathies.<sup>5–7</sup> Considering the importance and anxiety often associated with the potential risk of TAD in TOF patients with thoracic aortic aneurysm, we embarked on a population-based study to determine incidence of TAD in this population.

## Methods

We will make data, analytical methods, and study materials available to other researchers upon request. The National Inpatient Sample (NIS) is the largest all-payer database of

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## Clinical Perspective

### What Is New?

- Thoracic aortic dissection is uncommon in adults with tetralogy of Fallot.
- Risk factors for thoracic aortic dissection were male sex, older age, and hypertension.

### What Are the Clinical Implications?

- Tetralogy of Fallot patients with thoracic aortic aneurysm may require less-frequent imaging follow-up.
- Tetralogy of Fallot patients with thoracic aortic aneurysm who do not have risk factors for thoracic aortic dissection (male sex, older age, and hypertension) may benefit from conservative (nonsurgical) management considering the low event rate in this population.

hospital inpatient stays in the United States. The NIS contains discharge data from a 20% stratified sample of community hospitals and is a part of the Healthcare Cost and Utilization Project (HCUP), sponsored by the Agency for Healthcare Research and Quality.<sup>14</sup> Information regarding each discharge includes patient demographics, primary payer, hospital characteristics, principal diagnosis, up to 24 secondary diagnoses, and procedural diagnoses. The Mayo Clinic (Rochester, MN) Institutional Review Board approved this study, and informed consent was waived because the study is based on a review of a de-identified database.

Using the HCUP-NIS data from 2000 to 2014, adult patients (>18 years) admitted with a primary or secondary diagnosis of TOF (*International Classification of Diseases, Ninth Revision, Clinical Modification [ICD-9-CM] code 745.2*) were identified. Cases of TAD involving the ascending aorta were identified using *ICD-9-CM 441.01* and *441.1*. Patient characteristics (age, sex, race, socioeconomic status, and primary payer) and hospital characteristics (teaching status and location, bed size, and region) associated with each discharge were identified from the HCUP-NIS database. Deyo's modification of the Charlson Comorbidity Index was used to identify the burden of comorbid diseases (Table S1).<sup>15</sup> The primary outcome was to describe the incidence and risk factors for TAD in TOF patients.

As recommended by the HCUP-NIS, survey procedures using discharge weights provided with the HCUP-NIS database were used to generate national estimates. Categorical data are expressed as count (%), and continuous data are expressed as mean±SD or median and interquartile range for skewed data. Chi-square and *t* tests were used to compare categorical and continuous variables, respectively. Poisson regression was used to analyze trends of TAD-related admissions over the study duration. Risk factors for TAD were assessed using logistic

regression and are expressed as odds ratio (OR) and 95% CI. Two-tailed  $P<0.05$  was considered statistically significant. All statistical analyses were performed using SPSS software (version 25.0; IBM Corp, Armonk NY).

## Results

During the period between January 1, 2000 and December 31, 2014, there were an estimated 18 353 admissions in adults with TOF diagnosis, of which TAD of the ascending aorta (type A dissection) was diagnosed in 11 admissions (0.06%; 6 per 10 000 admissions). Mean age at the time of admission was higher in the TAD-related admissions compared with other admissions ( $49.8\pm 7.2$  versus  $38.4\pm 14.0$  years;  $P<0.001$ ). Table shows a comparison of the baseline characteristics of TAD-related admissions and other admissions. Incidence of TAD-related admissions was significantly higher in males compared with females (11.8 versus 1.0 per 10 000 admissions;  $P<0.001$ ) and higher in patients aged >60 years at time of admission (8.6 versus 5.9 versus 1.1 per 10 000 admissions;  $P<0.001$ ; Figure 1).

Of the 11 TAD-related admissions, 8 (73%) underwent aortic surgical intervention during the admission, and in-hospital mortality occurred in 5 of the 11 (45%) admissions. Risk factors associated with TAD-related admission were age >60 years (odds ratio, 2.41; 95% CI, 1.23–3.25;  $P=0.013$ ), male sex (odds ratio, 6.91; 95% CI, 4.85–8.54;  $P<0.001$ ) and hypertension (odds ratio, 1.74; 95% CI, 1.06–3.19;  $P=0.037$ ). With regard to hospital characteristics and resource utilization, TAD-related admissions were more likely to occur in large-bed-size hospitals (45%) compared with medium-bed-size hospitals (36%) and small-bed-size hospitals (18%;  $P=0.001$ ), and in urban nonteaching hospitals (55%) compared with urban teaching hospitals (46%) and rural hospitals (0%;  $P=0.004$ ). Mean length of hospital stay was  $6.9\pm 6.3$  days (4 days; interquartile range, 1–21).

## Discussion

Based on the review of a nationally representative database of hospital admissions in the United States, we identified 11 TAD-related admissions of 18 353 admissions in adults with TOF. All TADs were type A dissections. Incidence of TAD-related admissions was 6 per 10 000 admissions. Thoracic aortic aneurysm is common in adults with previous TOF repair, and prevalence is reported to range from 28% to 69%.<sup>1–4,8–10</sup> The wide variation in prevalence is because of differences in definitions of aneurysm used in these studies. Incidence of TAD after TOF repair is unknown, but it is generally considered to be uncommon. In a recent study of 453 adults with TOF followed at the Mayo Clinic, thoracic

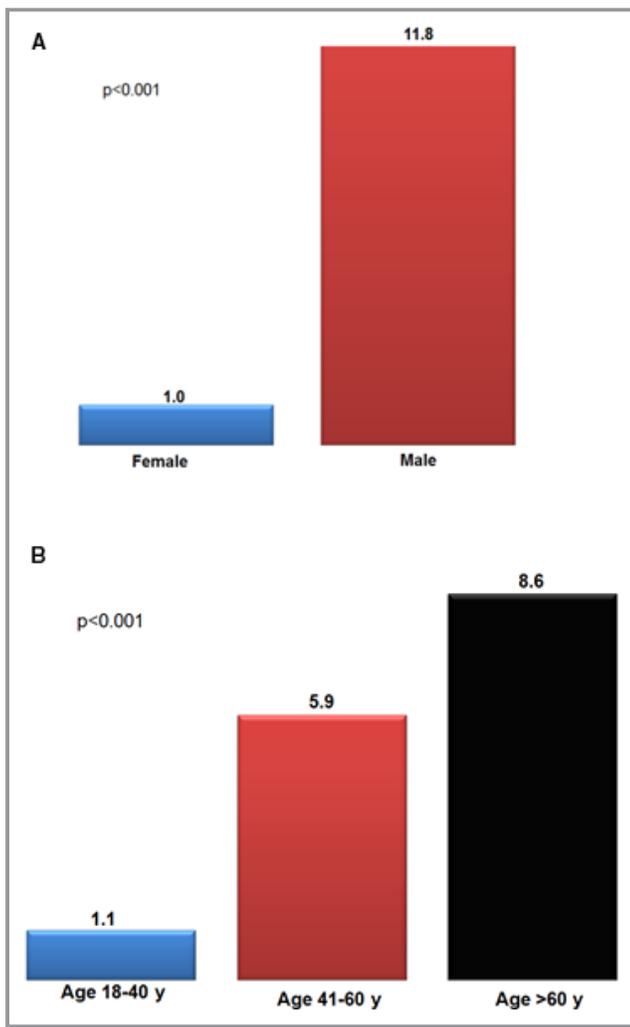
**Table.** Baseline Characteristics of Admissions With and Without TAD

Characteristic		TAD-Related Admission (N=11)	Others (N=18 342)	P Value
Age, y		49.8±7.2	38.4±14.0	<0.001
Female sex		1 (9.1)	9872 (53.8)	0.003
Race	White	10 (90.9)	10 225 (55.8)	0.001
	Black	1 (9.1)	1891 (10.3)	
	Hispanic	...	1658 (9)	
	Asian	...	408 (2.2)	
	Native American	...	67 (0.4)	
	Others	...	510 (2.8)	
	Missing	...	3583 (19.5)	
Primary payer	Medicare	3 (27.3)	5136 (28)	0.011
	Medicaid	4 (36.4)	4459 (24.3)	
	Private	4 (36.4)	7119 (38.8)	
	Uninsured	...	1004 (5.5)	
	No charge	...	103 (0.6)	
	Others	...	521 (2.8)	
Quartile of median household income for ZIP code	0 to 25th	4 (36.4)	4151 (22.6)	0.006
	26th to 50th	3 (27.3)	4591 (25)	
	51st to 75th	4 (36.4)	4588 (25)	
	75th to 100th	...	5012 (27.3)	
Hospital teaching status and location	Rural	...	1485 (8.1)	0.004
	Urban non-teaching	6 (54.6)	4353 (23.7)	
	Urban teaching	5 (45.5)	12 504 (68.2)	
Hospital bed size	Small	2 (18.2)	2203 (12)	0.001
	Medium	4 (36.4)	3618 (19.7)	
	Large	5 (45.5)	12 521 (68.3)	
Hospital region	Northeast	3 (27.3)	3801 (20.7)	0.004
	Midwest	4 (36.4)	3794 (20.7)	
	South	2 (18.2)	6731 (36.7)	
	West	2 (18.2)	4016 (21.9)	
Charlson Comorbidity Index		1.4±1.3	1.3±1.1	0.101
Comorbidities	Hypertension	6 (54.6)	3266 (17.8)	<0.001
	Hyperlipidemia	4 (36.4)	1258 (6.9)	<0.001
	Chronic kidney disease	2 (18.2)	974 (5.3)	0.03
	Atrial fibrillation	4 (36.4)	2802 (15.4)	0.001

Represented as percentage or mean±SD. TAD indicates thoracic aortic dissection.

aorta dilation was present in 69%, and severe aortic aneurysm defined as aortic dimension >50 mm was present in 9% of patients.<sup>16</sup> Total duration of follow-up in that study was 3700 patient-years, and there was no case of TAD observed during follow-up. Several other outcomes studies have reported similar findings of high prevalence of thoracic aortic aneurysm and zero incidence of TAD in the TOF population.<sup>1-4,8-10</sup>

To the best of our knowledge, there are only 4 cases of TAD in TOF patients reported in the literature. These TADs occurred in a 60-year-old man with aortic root dimension of 55 mm; a 36-year-old man with mid ascending aorta dimension of 93 mm; an 18-year-old man with 22q11 deletion and aorta root dimension of 70 mm; and a 30-year-old man with mid ascending aorta dimension of 71 mm.<sup>11-13,17</sup> All 4 cases were Stanford type A dissection. It is noteworthy who all these reported cases of TAD



**Figure.** Bar graph comparing incidence of TAD-related admissions, per 10 000 admissions by sex (A) and age group (B). TAD indicates thoracic aortic dissection.

occurred in males, similar to the sex distribution observed in the current study. In addition, 3 of the 4 reported cases had aortic dimensions that met elective replacement criteria, irrespective of the cause of aortic aneurysm.

In a cross-sectional study of 1181 patients with bicuspid aortic valve, prevalence of TAD was 5%, and ranged from 4% in patients with aortic dimension of 50 mm to 13% in patients with aortic dimension of 70 mm.<sup>18</sup> Other studies of degenerative and bicuspid aortic valve-related aortopathies have reported TAD prevalence of 2.5% to 7%.<sup>18–22</sup> In contrast to these studies, the estimated TAD incidence of 6 per 10 000 (0.06%) in the current study suggests an extremely low risk of TAD in TOF patients. Consistent with our speculation of low risk of TAD, none of the cohort studies of outcomes of aortic aneurysm in TOF patients have reported any TADs.<sup>1–4,8–10</sup> The only known cases of TAD in TOF patients are from case reports, and this does not provide an accurate estimate of risk (incidence) of TAD.

Another important finding from the current study was the striking sex difference in the incidence of TAD. Ten of the 11 cases of TAD occurred in males, yielding an incidence of 11.8 cases per 10 000 admissions compared with 1.1 per 10 000 in females. In addition to male sex, the other risk factors for TAD in this study were older age and hypertension. These risk factors are similar to the *traditional* risk factors for TAD in the general population, suggesting that TOF diagnosis per se does not confer any additional risk beyond these *traditional* risk factors for TAD.<sup>5,20,23</sup>

### Clinical Implications and Future Directions

The current study suggests a low risk of TAD in TOF patients, and this is consistent with data from previous cohort studies in TOF patients. Although the reason for the rarity of TAD is unknown, we speculated that this may be related to the presence of adhesions from previous sternotomies or may reflect a different underlying disease pathogenesis in TOF patients compared with other types of aortopathies. Our results have important clinical implications with regard to imaging surveillance and timing of surgical intervention. Prevalence of thoracic aortic aneurysm is higher in TOF patients compared with the general population; therefore, making clinical decisions based on outcomes data and practice guidelines derived from patients with degenerative and bicuspid aortic valve-related aortopathies may lead to *overtreatment*. Although surgical aorta replacement can be performed with very low surgical mortality in the high-volume centers,<sup>24</sup> the risks and benefits of this procedure should be carefully considered in patients with multiple previous sternotomies and high probability of future sternotomies for residual/recurrent hemodynamic lesions related to TOF. There is a need for in-depth genetic and phenotypic characterization of TOF patients with aortic aneurysm and TAD to better understand the pathobiology of this disease, and this can be achieved by creating a multicenter registry of aortic aneurysms and TAD in TOF patients. Appropriate identification and treatment of high-risk patients is critical because of the high mortality (46% in-hospital mortality) associated with TAD-related admissions.

### Limitations

The NIS is an administrative database and relies on accuracy of diagnosis codes. Second, we did not have data about thoracic aorta dimensions at time of admission, the incidence of bicuspid aortic valves and genetic syndromes in these patients, and other potential risk factors, such as rate of progression of aortic aneurysm and family history of TAD, in these patients. Finally, incidence of TAD may be underestimated in this study because the patients who had TAD and

died before arrival to the hospital would have been excluded from this study based on the sampling technique of the NIS. All these factors could have potentially influenced the observed findings in this study.

## Conclusions

In the current study, we reviewed data from 18 353 admissions in TOF patients and reported a low incidence of TAD of 6 per 10 000 admissions (0.06%). Risk factors for TAD were male sex, older age, and hypertension. The low event rate in this study suggests that using the practice guidelines for degenerative and bicuspid aortic valve-related aortopathies to decide on the timing of intervention may lead to *overtreatment* in this population because of the high prevalence of thoracic aortic aneurysm in TOF patients. Further studies are required to better understand the pathobiology of this disease in order to make evidence-based recommendations for surveillance and treatment.

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## Disclosures

None.

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# **SUPPLEMENTAL MATERIAL**

## **Data S1.**

### **Supplemental Introduction**

The experimental method was referring to our previous studies,<sup>1,2</sup> in which freshly isolated endothelial cells were obtained by wire-abrasion of upper-arm vein. However, this is the first study to use endothelial cells collected from radial catheter sheath. The non-invasive assessment enables us to measure and compare endothelial insulin resistance in many patients. Therefore, 1) validation of anti-phospho-eNOS antibody, and 2) optimization of quantifying immunofluorescence intensity should be reconfirmed. Furthermore, positive control and negative control were encouraged for validating the quantification of immunofluorescence intensity. For the reasons, we added the following data, which support the effectiveness of our method and the results.

In addition, we attached the solution path of the adaptive Lasso model to show the process of selecting the independent factors for the cardio-ankle vascular index (CAVI).

## **Supplemental Methods**

### **Endothelial cells**

Human umbilical vein endothelial cells (HUVECs) of passage 3-4 were cultured in dish or 4-well chamber slides. The cells were starved for 24 hours with serum-free medium. The cells were collected with lysate buffer for western blotting or fixed with 4% paraformaldehyde for immunofluorescence microscopy.

### **Western blot analysis**

Proteins were subjected to 4 to 12% gradient gels (Invitrogen) and transferred to polyvinylidene difluoride membranes. Membranes were initially blocked (Blocking one, Nacalai Tesque, Kyoto, Japan) for 1 hour. Membranes were cut and probed in blocking buffer containing primary antibodies of 1:2000 dilution: phosphorylated eNOS at serine 1177 (GeneTex, Irvine, CA), followed by the appropriate horseradish peroxidase–conjugated secondary antibody.

Immunoreactions were visualized with SuperSignal® West Dura Extended Duration Substrate (Thermo Scientific). Membranes were stripped (WB Stripping Solution, Nacalai Tesque) for 30 minutes at room temperature were

probed with phosphorylated eNOS at serine 1177 (1:1000 dilution; Cell Signaling, Danvers, MA), eNOS/NOS Type III (1:2000 dilution; BD Biosciences, San Jose, CA). The other cuts of membranes were probed with phosphorylated Akt at serine 473, total Akt [1:1000 dilution; Cell Signaling (CST)], GAPDH antibodies of 1:1000 dilution to verify equal protein loading. The bands were quantified by densitometry.

### **Immunohistochemistry**

The method was described in our previous paper.<sup>3</sup> Briefly, formalin-fixed saphenous veins were embedded in paraffin, and washed with xylene, 100% ethanol, 95% ethanol, and water for slide deparaffinization. Endogenous peroxidase was removed by treatment with 3% H<sub>2</sub>O<sub>2</sub> for 5 minutes and washing it under running water for 5 minutes. Antigen retrieval was performed by using the autoclave method (20 minutes at 121°C) and a citrate buffer (10 mmol/L, pH 8). After cooling, the plate was washed with phosphate-buffered saline (1.37 mol/L NaCl, 27 mmol/L KCl, 81 mmol/L Na<sub>2</sub>HPO<sub>4</sub>, 12 H<sub>2</sub>O, 14.7 mmol/L KH<sub>2</sub>PO<sub>4</sub>) and blocking was performed for 60 minutes using Blocking One (1/5, Nacalai Tesque). Incubation was performed overnight at 4°C with the primary

antibody (p-eNOS Ser1177, 1:50, GTX50212, GeneTex). After washing with phosphate-buffered saline, the secondary antibody (Histofine R Simple Stain MAX PO MULTI, Nichirei Bioscience Inc.) was reacted for 1 h. Finally, 3,3'-diaminobenzidine was added and allowed to react for 10 minutes, before the samples were dehydrated, penetrated, and re-sealed.

### **Assessment of protein expression by quantitative Immunofluorescence**

Fixed sample slides were thawed and rehydrated with PBS containing 50 mmol/L glycine (Sigma) for 10 minutes. The cells on the slides were permeabilized with 0.1% Triton X-100, and nonspecific binding sites were blocked with 0.5% BSA. The slides were incubated overnight at 4°C with primary antibodies against the following targets: p-eNOS Ser1177 (1:200 dilution; GeneTex). All of the slides were double-stained with an anti-von Willebrand Factor (vWF) antibody (1:300 dilution; Invitrogen, Carlsbad, CA), or eNOS/NOS Type III (1:200 dilution; BD Biosciences) for identification of endothelial cell. After the incubation, the slides were washed and incubated for 1 hour at 37°C with corresponding Alexa Fluor-488 and Alexa Fluor-594 antibodies (1:200 dilution; Invitrogen). The slides were washed again and

mounted under glass coverslips with Vectashield containing DAPI for nuclear identification (Vector Laboratories, Burlingame, CA).

Slide images of a fluorescence microscope at × 20 magnification were captured (KEYENCE, Osaka, Japan). Exposure time was constant, and image intensity was corrected for background fluorescence. Fluorescent intensity was quantified by a software (KEYENCE, Osaka, Japan). For each protein of interest, fluorescent intensity was quantified in 20 cells from each slide and averaged.

### **Making lentivirus vector expressing short-hairpin RNA to knock-down eNOS.**

The target sequence (GGAACAGCACAAGAGTTA) was designed from Human NOS3 mRNA sequence (NM\_000603.4). This sequence recognizes all isoforms of NOS3 and substantially (5 nt) differs from potential off-target sequences by Blast analysis. ShRNA expressing lentivirus was made as published by us before.<sup>4</sup>

## **Supplemental Results**

### **Validation of anti-phospho-eNOS antibody used in the study**

The method of this study is highly dependent on the quality of antibody. The western blot with anti-p-eNOS Ser1177 antibody (GTX50212, GeneTex), which were used for immunofluorescence in the study, exhibits a single band just below 150 kD (Figure S1A). The same membrane was stained with anti-p-eNOS Ser1177 antibody (CST #9571) (Figure S1B) and subsequently with anti-total-eNOS antibody (BD 610297) (Figure S1C). The bands located same molecular weight. Therefore, we confirmed that changes of immunofluorescent intensity come from the protein of this band. Thus, the anti-p-eNOS antibody (GTX50212) is available for quantification of immunofluorescent intensity.

Figure S2 shows the sections of a human saphenous vein harvested from a same patient during coronary artery bypass grafting. The intima was stained by anti-p-eNOS antibody (GTX50212) (left) as same as anti-total-eNOS antibody (BD 610297) (right). The image of vascular endothelial growth factor (VEGF)-stimulated section is also available in our recent paper.<sup>3</sup> The intensity was thickened by 30 minutes stimulation of VEGF.

### **Optimization of quantifying immunofluorescence intensity**

HUVECs were cultured in 4-well chamber slides (Figure S3A). The cells were starved for 24 hours with serum-free medium and fixed with 4% paraformaldehyde at each time point after stimulation. From the results, the timing of evaluating immunofluorescence intensity was considered appropriate at 30 minutes for insulin, VEGF, and 15 minutes for ACh (Figure S3B).

Figure S4A shows the immunoblotting of the HUVECs with p-eNOS Ser1177 (GTX50212). eNOS was activated after increase of p-Akt Ser473 (CST #4060). The comparison between western blotting and immunofluorescence is shown in Figure S4B. The inter-class correlation was 0.928 ( $p = 0.004$ ). The result indicates that the quantification of immunofluorescent intensity is functional to detect the change of p-eNOS Ser1177.

### **Validation of method with eNOS knockdown HUVECs**

We created HUVECs with eNOS knock-down (KD) for negative controls to test our assessment of immunofluorescent microscopy. The images of HUVECs after insulin stimulation treated by KD were shown in Figure S5A. The total

eNOS (red) was reduced by KD. The p-eNOS Ser1177 (GTX50212, green) was extinguished regardless of insulin stimulation.

Figure S5B shows the western blotting of HUVECs with or without KD. The bands of p-eNOS Ser1177 (GTX50212) was eliminated by KD. Thus, these results reconfirmed the validation of the antibody.

### **Positive control with serum-stimulation**

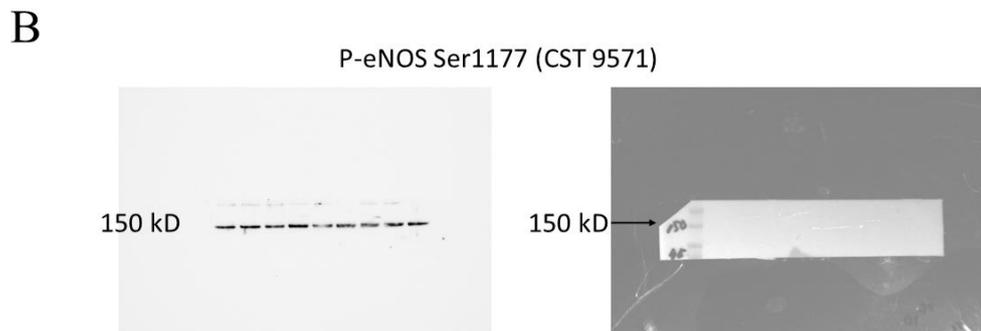
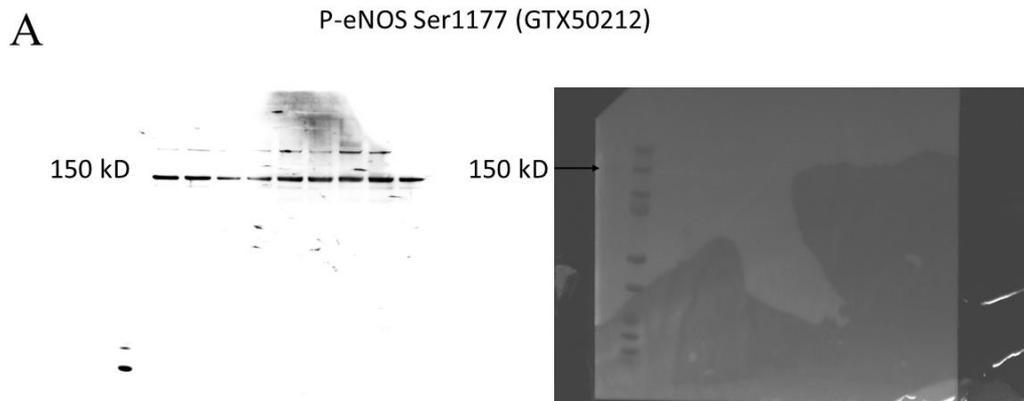
It was previously known that thrombin increases eNOS activation.<sup>5-7</sup> Figure S6A shows the increased immunofluorescent intensity of p-eNOS Ser1177 by culturing with human fresh serum for 30 minutes before fixation. The effect was not seen in the HUVECs treated by KD. The western blotting confirmed that p-eNOS Ser1177 increased by serum after augmentation of p-Akt Ser473 (Figure S6B)

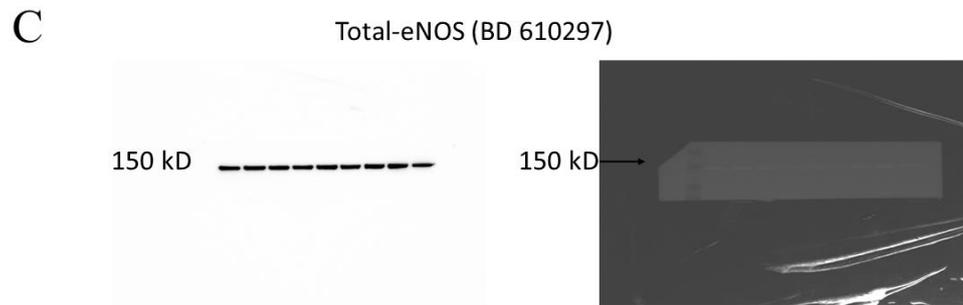
. This serum-stimulated increase of p-eNOS Ser1177 was absent by KD (Figure S6C) as same as other stimulations (Figure S6D). Therefore, we applied serum-stimulation to freshly isolated arterial endothelial cells as a positive control. The results were described in the main text (Figure 3).

**The supplemental data of the adaptive Lasso regression for CAVI.**

Figure S7 shows the solution path of the adaptive Lasso regression model (model 3 in Table 4) described by JMP pro. version 13.1.0 (SAS Institute Japan, Tokyo).

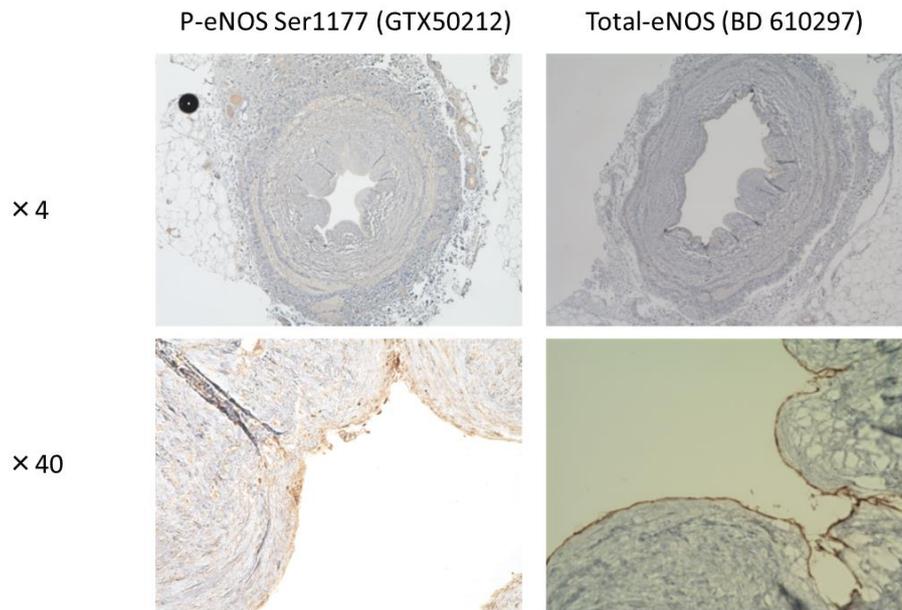
**Figure S1. The validation of anti-p-eNOS antibody by western blotting.**





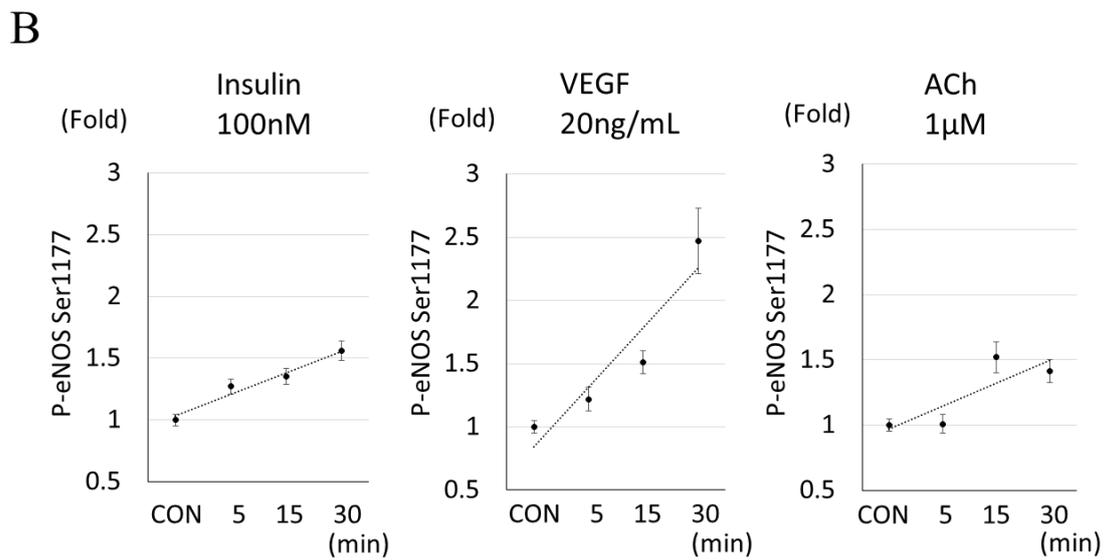
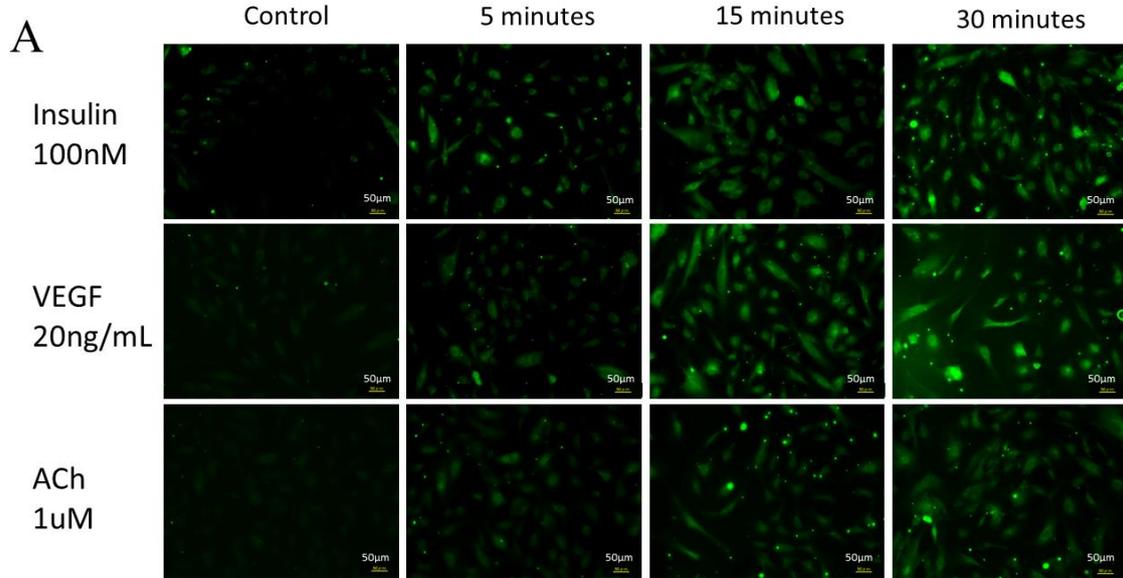
A: The western blot with anti-p-eNOS Ser1177 antibody (GTX50212), B: anti-p-eNOS Ser1177 antibody (CST #9571), C: anti-total-eNOS antibody (BD 610297). The figures are a chemifluorescent image (left) and a digitizing image (right).

**Figure S2. The validation of anti-p-eNOS antibody by immunohistochemistry.**



The images show the location probed by the anti-p-eNOS Ser1177 antibody (GTX50212) (left) and anti-total-eNOS antibody (BD 610297) (right).

**Figure S3. Optimal timing for evaluating immunofluorescent intensity.**

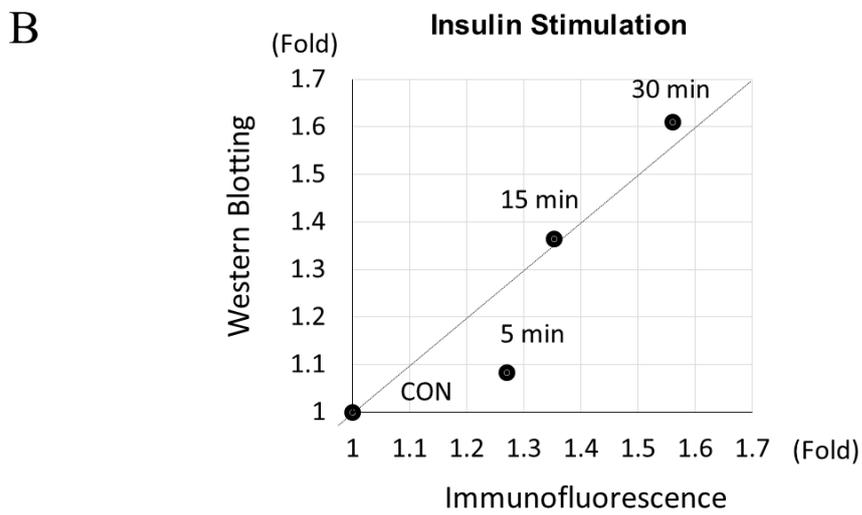
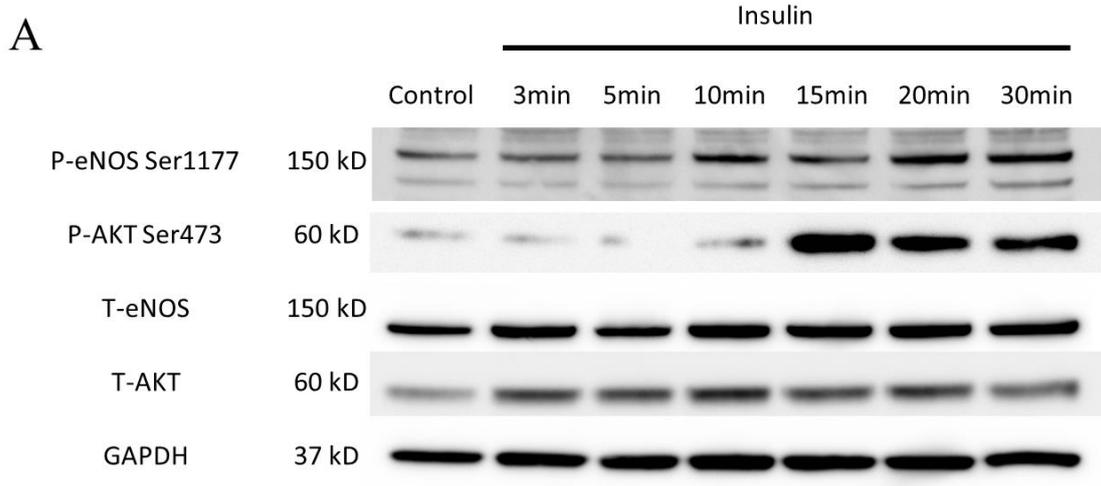


A: The immunofluorescent images with anti-p-eNOS antibody (1:200, GTX50212). Control slides had no stimulation. The times after each stimulation were indicated above the pictures.

B: The graphs of the intensities (ratio to average of control, mean  $\pm$  standard error).

VEGF, vascular endothelial growth factor; ACh, acetylcholine.

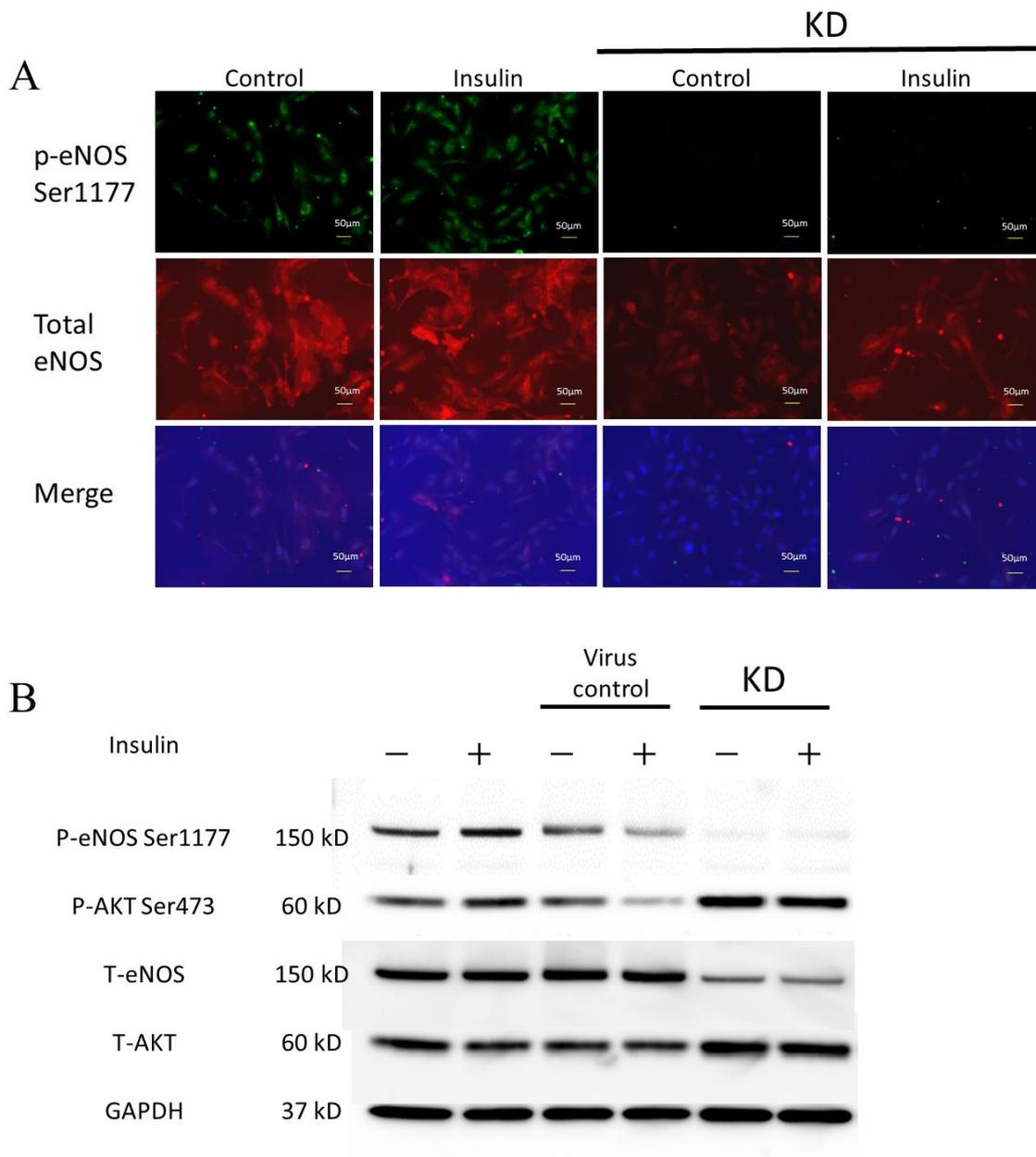
**Figure S4. The relationship between intensities of western blotting and immunofluorescence of HUVECs after insulin-stimulation.**



The immunoblotting image (A) shows gradual increase of p-eNOS Ser1177 after rise of p-Akt Ser473 by addition of insulin 100 nM. The plots (B) shows the

positive correlation of the results from western blotting and immunofluorescence.

**Figure S5. Elimination of p-eNOS Ser1177 by eNOS knockdown.**

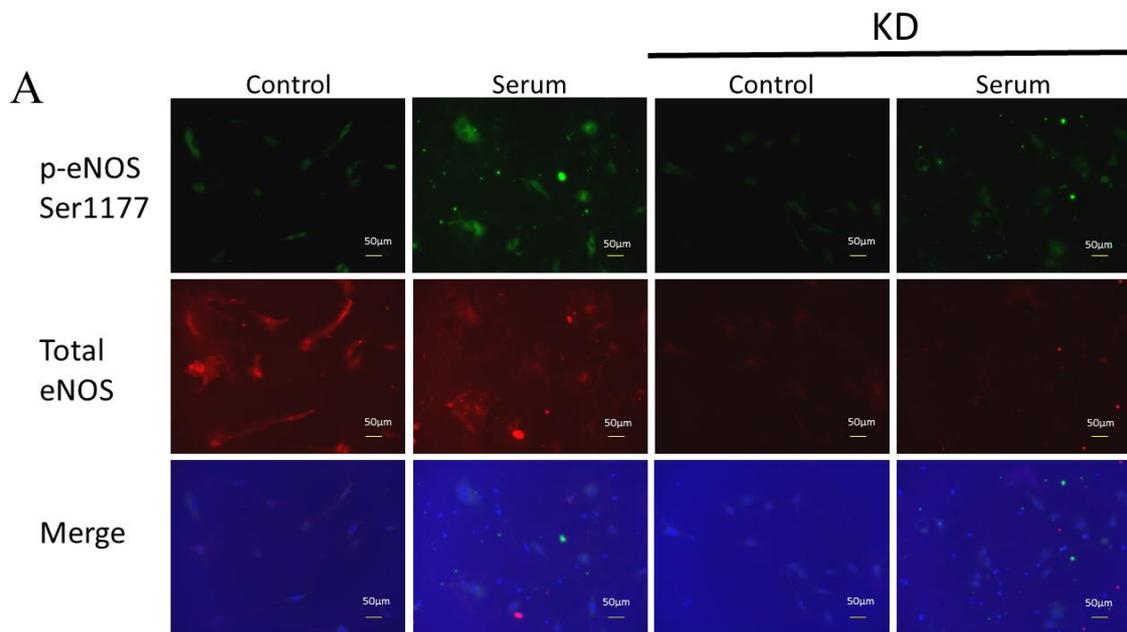


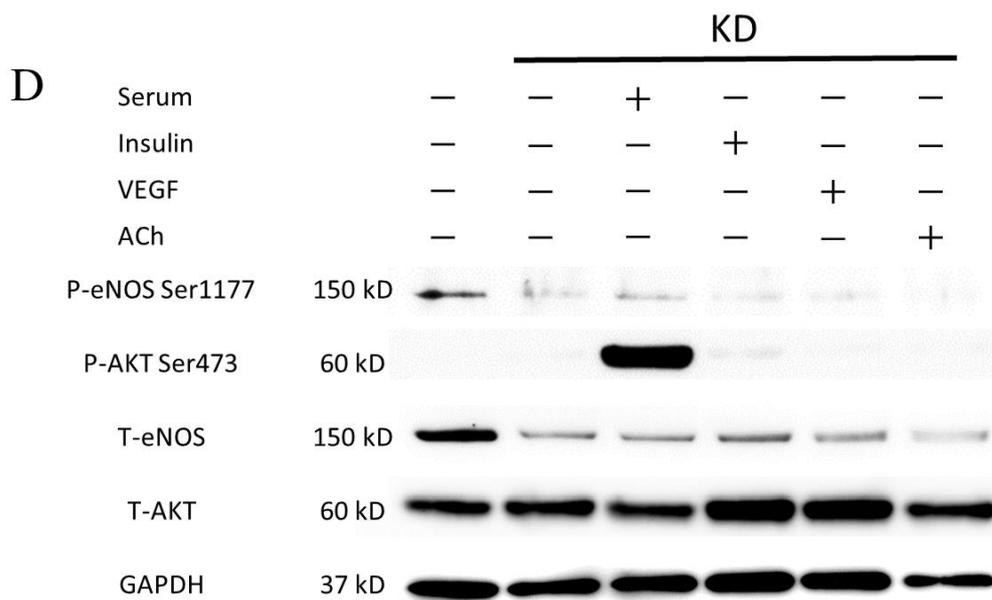
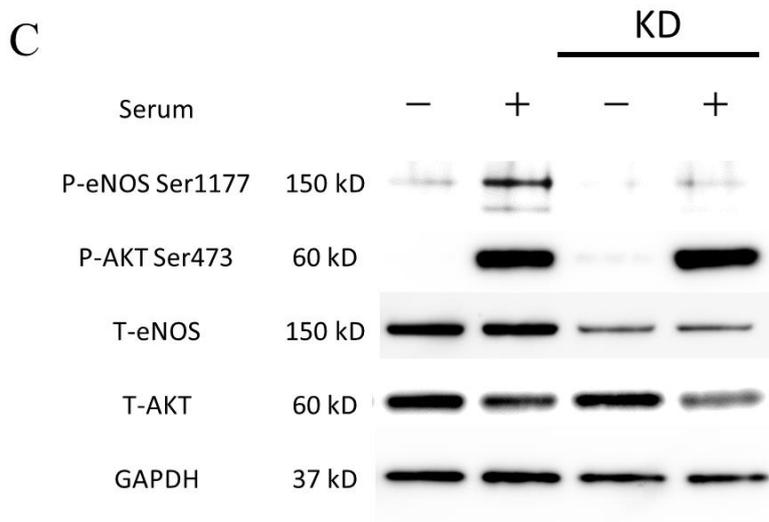
A: The immunofluorescent images with anti-p-eNOS antibody (GTX50212). B:

The western blotting. Control slides had no stimulation. Insulin stimulation was

100 nM 30 minutes. KD, eNOS knockdown.

**Figure S6. Augmentation of p-eNOS Ser1177 by serum-stimulation.**





A: The immunofluorescent images with anti-p-eNOS antibody (GTX50212). B:

The western blotting of time-course p-eNOS Ser1177 after serum-stimulation.

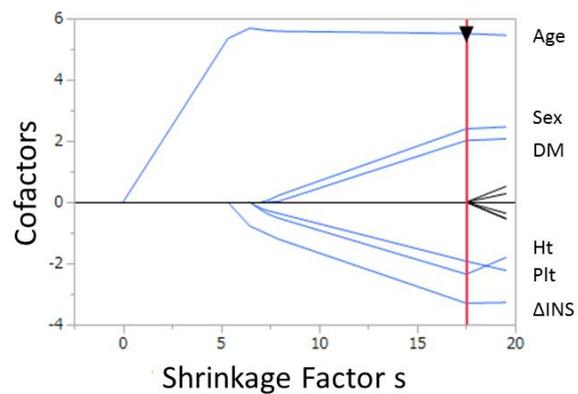
C: The western blotting showing the effects of KD on HUVECs with serum-

stimulation. D: The western blotting showing the effects of KD on HUVECs with other stimulations.

Control slides had no stimulation. Serum-stimulation was incubating with human fresh serum for 30 minutes. Insulin-stimulation was addition of insulin 100 nM for 30 minutes. VEGF-stimulation was 20 ng/mL for 30 minutes. ACh-stimulation was 1  $\mu$ M for 15 minutes.

KD, eNOS knockdown; VEGF, vascular endothelial growth factor; ACh, acetylcholine.

**Figure S7. Solution path.**



The original prediction formula was as follows;  $7.347962 + (-0.015367) \cdot \Delta\text{INS} + 0.0831406 \cdot \text{Age} + 0.869302 \cdot \text{Sex} + 0.6601768 \cdot \text{DM} + (-0.086475) \cdot \text{Ht} + (-0.043499) \cdot \text{Plt}$ .

The categorical variables are Sex (man 1, woman 0) and DM (yes 1, no 0).

$\Delta\text{INS}$ , percent change in insulin-induced p-eNOS at Ser1177; DM, diabetes mellitus; Ht, hematocrit; Plt, platelets.

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