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Pericardial Adipose Tissue Thrombospondin-1 Associates with Anti-angiogenesis In Ischemic Heart Disease

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Abstract

Accumulation of ectopic pericardial adipose tissue has been associated with cardiovascular complications which, in part, may relate to adipose-derived factors that regulate vascular responses and angiogenesis. We sought to characterize adipose tissue microvascular angiogenic capacity in individuals undergoing elective cardiac surgeries including aortic, valvular, and coronary artery bypass grafting. Pericardial adipose tissue was collected intraoperatively and examined for angiogenic capacity. Capillary sprouting was significantly blunted (2-fold, $p<0.001$) in subjects with CAD (age 60±9 years, BMI 32±4 kg/m^2, LDL-C 95±46 mg/dl, n=29) compared to age-, BMI-, and LDL-C matched individuals without angiographic obstructive CAD (age 59±10 years, BMI 35±9 kg/m^2, LDL-C 101±40 mg/dl, n=12). For potential mechanistic insight, we performed mRNA expression analyses using quantitative RT- PCR, and observed no significant differences in pericardial fat gene expression of pro-angiogenic mediators vascular endothelial growth factor-A (*VEGF-A*), fibroblast growth factor-2 (*FGF-2*), and angiopoietin-1 (*angpt1*), or anti-angiogenic factors soluble fms-like tyrosine kinase-1 (*sFlt-1*) and *endostatin*. In contrast, mRNA expression of anti-angiogenic thrombospondin-1 (*TSP-1*) was significantly upregulated (2-fold, $p=0.008$) in CAD compared to non-CAD subjects, which was confirmed by protein western-immunoblot analysis. TSP-1 gene knockdown using shRNA lentiviral delivery significantly improved angiogenic deficiency in CAD ($p<0.05$). In conclusion, pericardial fat in subjects

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Declaration of interests
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Disclosures
The authors declare no conflicts of interest.

Consent to Participate
Study participants were informed about the design and objectives of the study, and all included subjects read and signed the informed consent form for participation.

Consent for Publication
All subjects signed informed consent including permission for publication of the results.

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with CAD may be associated with an anti-angiogenic profile linked to functional defects in vascularization capacity. Local paracrine actions of TSP-1 in adipose depots surrounding the heart may play a role in mechanisms of ischemic heart disease.

Keywords

Coronary artery disease; angiogenesis; pericardial adipose tissue; obesity; coronary artery bypass grafting

Introduction

Excessive build-up of ectopic adipose tissue surrounding the heart has been associated with cardiovascular complications, but mechanisms are incompletely understood. Accumulation of increased pericardial adipose tissue (PAT), in particular, has been linked to incident heart failure and coronary artery disease (CAD), independent of conventional risk factors.[1,2] Pericardial adipose tissue has been shown to exhibit pro-inflammatory characteristics[3] and immune dysregulation in PAT has been linked to the prevalent CAD. [4] Pericardial adipose depots also harbor cells of vascular origin, and whether differential phenotypes in microvascular regenerative capacity exist in the pericardial adipose tissue of humans has not been previously examined. Thus, our primary objectives were to characterize angiogenic properties of PAT in subjects with and without ischemic heart disease, and test the hypothesis that imbalances in pro- and anti-angiogenic mediators may identify potential regulators of angiogenesis that could play a role in myocardial ischemia.

Methods

Study population

Forty-one consecutive men and women undergoing elective cardiothoracic surgery at Boston Medical Center were recruited into the study. Surgeries were coronary artery bypass grafting (CABG, n=29), aortic valve replacement (AVR, n=5), mitral valve replacement (MVR, n=4), ascending aortic aneurysm repair (n=2), and unroofing of a coronary myocardial bridge (n=1). Subjects undergoing non-CABG surgeries (n=12) all had no obstructive CAD by angiography. Prior to surgery, clinical characteristics, including age, BMI, hypertension prevalence, and diabetes mellitus history were recorded. Fasting blood samples were drawn and analyzed for total cholesterol, LDL-C, HDL-C, triglycerides, HbA1c, and creatinine levels, and aliquots stored at −80°C for further studies. During the cardiovascular operation, adipose tissue specimens were collected from the anterior pericardium and processed immediately. Pericardial fat was defined as adipose tissue located outside (external to) the fibrous pericardium from which samples were collected, [4,5] and distinct from epicardial fat that is the tissue layer situated between the myocardium and visceral pericardium, which was not examined. Specimens were procured using scissors rather than electrocautery to avoid thermal tissue damage. Pregnant individuals were not eligible for surgery and thus excluded. Left ventricular ejection fraction was derived from clinical echocardiograms. The study was approved by the Boston University Medical Center Institutional Review Board, and written consent was obtained from all participants.

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Fat pad angiogenesis assays

Pericardial adipose tissue samples collected during cardiothoracic surgery were immediately placed in sterile EBM-2 Endothelial Cell Basal Medium-2 (EBM-2; Lonza, cat # CC-3156). Adipose tissues were minced with scissors and digested with 1 mg/mL collagenase type II (Worthington Biochemical, cat # LS004176) for 15–30 minutes at 37° C in a shaking water bath. The tissues were strained in a 100μm nylon filter and washed once with EBM-2 media. Specimens of 1–2 mm^2 were embedded in 200μL per well Matrigel (Corning, cat # 354230) in 48-well plates on ice and incubated for 45 min at 37° C for polymerization. Following polymerization, 0.5mL media was added per well, and media was replaced on days 2, 4, and 6. Angiogenic sprouts growing from the central fat pad were quantified on day 7 along the periphery using a10x magnification microscope, as previously described. [6]

TSP-1 silencing using shRNA

Pericardial adipose tissue specimens from subjects with CAD were transfected with TSP-1 silencing shRNA lentiviral particles (Santa Cruz, sc-36665-V) or negative control shRNA Lentiviral Particles-A without TSP-1 silencing (Santa Cruz, sc-108080) according to the manufacturer's protocol. Lentivirus particles 1×10^5/ml were added to Matrigel before tissue implantation. Collagenase-digested tissues were embedded in 60μL Matrigel per well in a 96-well plate and then polymerized for 45min at 37° C. Following polymerization, 0.2mL media per well was added with 1×10^5/ml lentivirus particles and 5ug/ml polybrene for 3 days. Angiogenic sprouts growing from the central fat pad were quantified on day 7 along the periphery using a 10x magnification microscope, and tissues were stored for gene expression. To assess cell viability for each condition, we employed the trypan blue hemocytometer methodology.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Adipose tissue samples were collected in an RNA-stabilizing solution (Ambion, cat#AM7024) and preserved at –80 °C until RNA isolation. Total mRNA was isolated using the RNeasy Lipid Tissue Mini Kit (Qiagen, cat# 74804). RNA was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, cat# 4368814) and pre-amplified using TaqMan™ PreAmp Master Mix Kit (Applied Biosystems, cat# 4384267). Quantitative PCR was performed for the expression of vascular endothelial growth factor-A (*VEGF-A*), fibroblast growth factor-2 (*FGF-2*), and angiopoietin-1 (*Angpt1*), soluble fms-like tyrosine kinase-1 (*sFlt-1*), endostatin (*COL18A1*), thrombospondin-1 (*TSP-1*), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) genes (Table 1) using iTaq™ Universal Probes Supermix (BIO-RAD, cat# 1725134) in ViiA™ 7 Real-Time PCR System. Expression data for all target genes were normalized to *GAPDH*, analyzed using the Ct method, and expressed as a fold difference of expression in specimens from non-CAD subjects.

Western Immunoblot Analyses

Proteins were extracted from frozen adipose tissue by homogenization in liquid nitrogen in 1X lysis buffer (Cell Signaling, cat#9803) with a protease and phosphatase inhibitor cocktail (Thermo Scientific, cat#78440). Protein was measured using Bradford's method

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and normalized. 10 μg of total protein was subjected to electrophoresis using SDS-polyacrylamide gel and blotted to nitrocellulose membranes using the Bio-Rad Transblot Turbo Transfer system. The membranes were blocked with 5% bovine serum albumin in TBST and incubated overnight at 4°C with primary antibodies to thrombospondin-1 and GAPDH (1:1000 dilution; Sigma, St Louis, MO). The membranes were washed three times and incubated on a shaker at room temperature for 1 h with horseradish peroxidase (HRP)-conjugated secondary antibody. After washing, chemiluminescence (Pierce™ ECL Western Blotting Substrate, Thermo Scientific™, cat#32209) was used to detect HRP enzyme activity. Densitometric analysis of bands was performed using ImageQuant™ LAS 4000 biomolecular imaging system (GE Healthcare, Pittsburg, PA). Protein levels were quantified using the Image Studio Lite 5.2 software, and values were normalized to GAPDH.

Enzyme-linked immunosorbent assay (ELISA)

Total protein was measured from previously collected plasma in a subset of subjects with available samples for analyses. Soluble thrombospondin-1 concentration was measured using commercially available ELISA kits (R & D systems, cat# DTSP10) according to the manufacturer's instructions and normalized to total protein. Data were expressed as a fold difference compared to the non-CAD group.

Statistical Analysis

Data are expressed as the mean ± SD in tables. Student's unpaired t-tests and one-way ANOVA were used to compare angiogenic capacity and clinical parameters, including plasma TSP-1 concentrations between groups that were normally distributed. Non-parametric Mann-Whitney tests were used to compare group differences in gene and/or protein expression that were non-normally distributed, as assessed by Kolmogorov-Smirnov methodology. The Holm-Sidak correction was performed for any potential multiple testing in gene expression analyses. Chi-square tests were used to compare frequency proportions. Regression analyses were performed with angiogenesis as the dependent variable in subjects with and without CAD, and clinical covariates including age, BMI, LDL-cholesterol, triglycerides, HbA1c, diabetes mellitus, hypertension, and LVEF. Graphic data are presented as mean ± SEM unless otherwise indicated. A value of $p < 0.05$ was considered significant for all analyses.

Results

Clinical Characteristics

A total of 41 subjects were recruited for the study. Clinical characteristics of all subjects are displayed in Table 2, which comprised of individuals with CAD undergoing CABG (n=29) compared to non-CAD populations that included AVR (n=5), MVR (n=4), ascending aortic aneurysm repairs (n=2), and unroofing of a myocardial bridge (n=1). Seventy three percent of subjects were male. There were no significant differences in age, BMI, total cholesterol, and LDL-C between subjects with vs. without obstructive CAD. Individuals with CAD had a slightly lower left ventricular ejection fraction and higher prevalence of diabetes mellitus and HbA1c levels.

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Microvascular angiogenic capacity is reduced in ischemic heart disease

Angiogenic sprouting capacity quantified *ex-vivo* for up to 7 days was diminished in the pericardial fat tissue of subjects with CAD by approximately 2-fold, compared to individuals without CAD (Figures 1a and c, p<0.001). The extent of angiogenic impairment was not different between subjects with and without concomitant diabetes mellitus (Figure 1b). Regression analyses using clinical covariates did not demonstrate any significant associations independent of CAD. P values for all variables tested were as follows: age (p=0.14), BMI (p= 0.17), LDL cholesterol (p=0.71), triglycerides (p=0.7), HbA1c (p=0.31), DM (p=0.5), HTN (p=0.59) and LVEF (p=0.9).

Quantitative PCR, Western immunoblot, and ELISA analyses

We examined the expression of several transcripts of interest that have been shown to be key regulators of angiogenic processes. As shown in Figure 2 (2a, 2b, and 2c), there were no significant differences in pro-angiogenic *VEGF-A, FGF-2*, or A*ngpt-1* in the PAT of subjects with vs. without CAD. [7–9] Similarly, no significant differences were observed in anti-angiogenic *endostatin* and *sFlt-1* expression (Figures 2e and 2f). [10–12] In contrast, anti-angiogenic *TSP-1* expression was significantly upregulated by over 2-fold in the pericardial fat of CAD subjects (p=0.008, Figure 2d). TSP-1 mRNA expression was confirmed at the protein level by immunoblot analysis (p<0.05, Figure 3a). In contrast, there were no differences in plasma concentrations of circulating TSP-1 between subjects with vs. without CAD (Figure 3c) in exploratory studies in a subset of individuals with available blood samples for analyses.

TSP-1 antagonism augments angiogenic capacity in CAD subjects

To examine the pathophysiological significance of TSP-1, we employed a shRNA lentiviral delivery approach *ex vivo* to downregulate its expression in the adipose tissue of subjects with CAD. As shown in Figure 4, shRNA targeting TSP-1 significantly reduced its gene expression in PAT (**4a**) and was associated with an improvement in angiogenic responses (**4b**) in subjects with CAD (p<0.05). We also examined cell viability using a trypan blue hemocytometer and observed no significant differences with 95% viability for each condition.

Discussion

In the present study we demonstrate, to our knowledge for the first time, reduced angiogenic capacity in the pericardial adipose tissue of subjects with CAD compared to individuals without ischemic heart disease, independent of age, BMI or LDL-C levels. We identified tissue up-regulation of anti-angiogenic TSP-1 at mRNA and protein levels in subjects with CAD, while typical pro-angiogenic factors including VEGF-A were not differentially expressed. Lentiviral TSP-1 silencing induced recovery of vascularization capacity providing evidence for functional plasticity of human pericardial adipose tissue. The findings also raise the possibility that anti-proliferative signaling in adipose depots surrounding the heart may play paracrine roles in mechanisms of cardiovascular disease.

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Pericardial adipose tissue is a metabolically active endocrine depot that overlies the external surface of the fibrous pericardial layer, with similar embryonic origins as visceral fat, supplied by non-coronary vasculature, [13] and anatomically distinct from epicardial fat. [5] Pericardial adipose can comprise up to 70% of total cardiac fat, and has been shown to correlate with intra-abdominal visceral fat volume, metabolic syndrome components, insulin resistance, and coronary artery calcifications, [14–20] [21] and is frequently also termed paracardial fat. The accumulation of both epicardial and pericardial fat around the heart, that share similar transcriptional profiles, have been linked to cardiovascular risk. [1,20,22] PAT is emerging as an independent parameter of cardiovascular risk and has been associated with coronary atherosclerosis, [1,23,24] myocardial dysfunction, [25–27] and incident heart failure, [28] however mechanisms remain poorly understood. It is recognized that dysfunctional fat has the capacity to release an array of factors that can negatively modulate vascular and myocardial function, and has been associated with inflammation and oxidative stress. [4,29] A prevailing hypothesis is that the pericardium may be exerting paracrine regulatory control over adjacent coronary vascular beds. Microarray studies show that in subjects with CAD, pro-inflammatory signatures are upregulated in pericardial fat compared to subcutaneous adipose tissue [22] and individuals without CAD. [4] While the angiogenic profile of PAT has not been studied extensively, its ability to express angiogenic effectors has been examined in the context of potential therapeutic use. [30] Resident stem cells collected from adipose tissue surrounding the heart are capable of regulating microvascular endothelial function *ex vivo*, [31] and mesenchymal stromal cells derived from the PAT of cardiac surgery patients display angiogenic potential. [32] Moreover, we analyzed available sequencing data in a published smaller cohort (n=5, GSE179397) and found increased expression of TSP-1 by nearly 1.7-fold in the PAT of patients with CAD (Ensembl ID ENSG00000137801). [4] Our results are concordant with those data, and in addition we presently demonstrate functional deficits in angiogenic capacity in conjunction with TSP-1 overexpression.

TSP-1 is a multi-domain matrix glycoprotein which can interact with numerous ligands including structural components of extracellular matrix, growth factors, receptors, proteases, and cytokines. [33] TSP-1 has been shown to be a natural inhibitor of neovascularization, [34,35] acting through the CD36/p38 MAPK mitogen signaling pathway that promotes endothelial cell apoptosis [36] and the CD47/cGMP pathway that leads to inhibition of angiogenesis. [37] TSP-1 also hinders vascular proliferation by acting as a scavenger for FGF-2 and VEGF-A. [38,39] TSP-1 can upregulate monocyte binding, promote TGF-β expression, and inhibit nitric oxide signaling [40] to support atherosclerosis, and patients with acute coronary syndromes display elevated plasma TSP-1 levels. [41] Growing evidence suggests that TSP-1 plays a role in various cardiovascular disorders and has been considered as a therapeutic target. [33]

Reparative angiogenesis is a fundamental physiological process that involves growth of new blood vessels from preexisting networks that is essential for tissue recovery following ischemic injury both in CAD and peripheral arterial disease. Angiogenic insufficiency impairs vascular collateralization and worsens ischemia. [42,43] As such, there has been great interest in therapeutic angiogenesis to salvage ischemic damage, although most trials involving growth factor delivery in the form of proteins or genes encoding targets such as VEGF and FGF to date with have been largely negative. [44] While our manuscript does not directly address this issue, it touches upon the notion that perhaps the therapeutic focus

could be on anti-angiogenic factors rather than pro-angiogenic monotherapies. Upregulated TSP-1 expression is manifest in the heart, vasculature, and kidneys of diabetic and obese individuals as well as experimental rodent models. [45–47] Preclinical work has demonstrated that TSP-1 deletion in mice protects against leptin-induced atherosclerosis. [48] Collectively, these data underscore TSP-1 as a potential target for mitigating vasculopathy that deserves consideration.

There are several limitations of our study to consider. First, our findings are observational and do not provide a causal role of TSP-1 in ischemic heart disease. Second, the precise role of pericardial fat in regulating the coronary vasculature remains undefined. Conceptually, it is possible that abnormalities in PAT stem partly from "inside-out" atherosclerotic signaling that extends out to surrounding adipose tissue rather than vice versa. However anti-angiogenic upregulation locally would be counterintuitive under conditions of ischemia. Third, the primary driver of TSP-1 overexpression in subjects with CAD remains unclear. Fat depots regulate their expansibility via cross-talk with resident progenitor and endothelial cells, and adipose tissue dysfunction commonly seen in obesity may be associated with microvascular dysfunction.[49] Fourth, *ex vivo* assessment of angiogenic capacity may not recapitulate the *in vivo* microenvironment. However, we point out that we utilized freshly isolated samples from living subjects rather than rely on autopsy specimens. Moreover, we provide direct pathophysiological evidence of TSP-1 control of vascularization capacity in tissues surrounding the human heart that has not been previously described. Lastly, we did not find up-regulation of TSP-1 in the plasma of CAD subjects which may, in part, relate to our small sample size that may be underpowered, and also suggests that local paracrine actions may be tissue-specific.

In conclusion, pericardial fat in subjects with CAD is associated with increased TSP-1 expression that is linked to functional defects in angiogenic potential. Local paracrine actions of TSP-1 in adipose depots surrounding the heart may play a role in mechanisms of ischemic heart disease that warrants additional investigation.

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Nonstandard Abbreviations and Acronyms:

Angpt1	angiopoietin-1
ApoE	apolipoprotein E
a.u.	arbitrary units
AVR	aortic valve replacement

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BMI	body mass index
CABG	Coronary artery bypass grafting
CAD	coronary artery disease
CD47/cGMP	Cluster of Differentiation 47/ cyclic guanosine monophosphate
CD36/p38 MAPK	Cluster of Differentiation 36/p38 mitogen-activated protein kinases
COL18A1	collagen type XVIII alpha 1 chain
EAT	epicardial adipose tissue
EBM-2	EBMTM-2 Endothelial Cell Basal Medium-2
ELISA	enzyme-linked immunosorbent assay
FGF-2	fibroblast growth factor-2
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HbA1c	Hemoglobin A1c
HDL-C	high-density lipoprotein cholesterol
hMSC	human mesenchymal stromal cells
HRP	horseradish peroxidase
LDL-C	low-density lipoprotein cholesterol
MVR	mitral valve replacement
PAT	pericardial adipose tissue
PCR	polymerase chain reaction
RT-qPCR	real-time quantitative PCR
sFlt-1	soluble fms-like tyrosine kinase-1
shRNA	short hairpin RNA
TBST	Tris-buffered saline with Tween
TSP-1	thrombospondin-1
VEGF-A	vascular endothelial growth factor-A.

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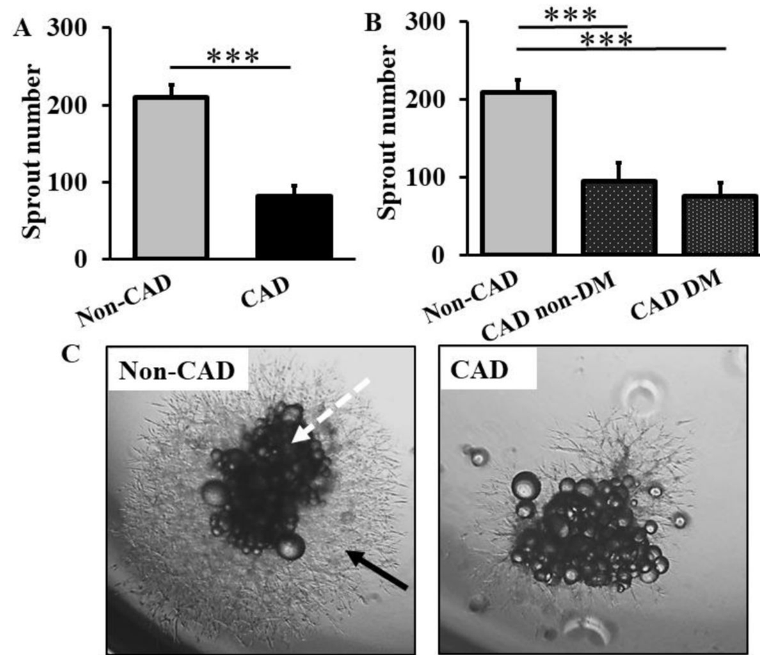

Figure 1: CAD is associated with reduced angiogenic capacity in pericardial adipose tissue.
a) PAT angiogenic capillary growth is blunted in subjects with CAD compared to the non-CAD group (***P<0.001). **b)** In subjects with CAD, angiogenic impairment was similar in individuals with and without DM (p=NS by ANOVA). **c)** Representative images of preserved angiogenic growth in non-CAD subjects (left panel) compared to blunted proliferation in CAD (right panel). The solid black arrow represents capillary networks emanating from the central fat pad which is indicated by the dashed white arrow (non-CAD, n=11; CAD, n=25). Data are presented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.0001).

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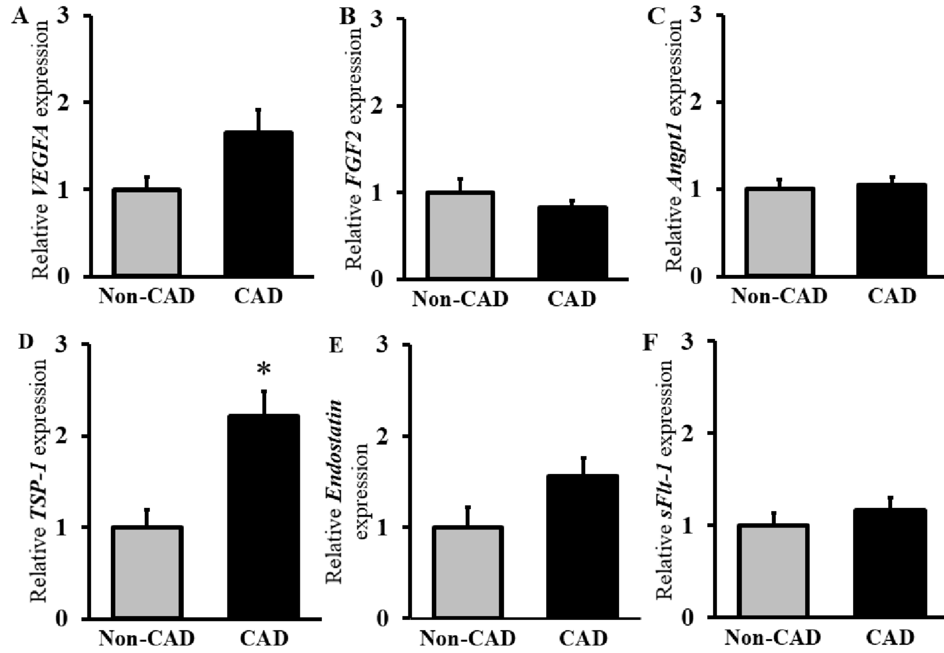

Figure 2: Expression of pro-and anti-angiogenic genes in PAT in subjects with CAD compared to without CAD.

There were no significant differences in pro-angiogenic gene expression for **a**) *VEGF-A,* **b**) *FGF2*, or **c**) *Angpt1* (p=NS). However, anti-angiogenic **d**) TSP-1 was significantly upregulated in subjects with CAD (*p=0.008), whereas no significant differences in **e**) endostatin or **f**) sFlt-1 were observed (p=NS). Analyses were performed by qPCR and normalized to *GAPDH.* Data are expressed as relative fold-difference compared to non-CAD subjects indexed to 1 (non CAD, n=12; CAD, n=25). Data are displayed as mean ±SE.

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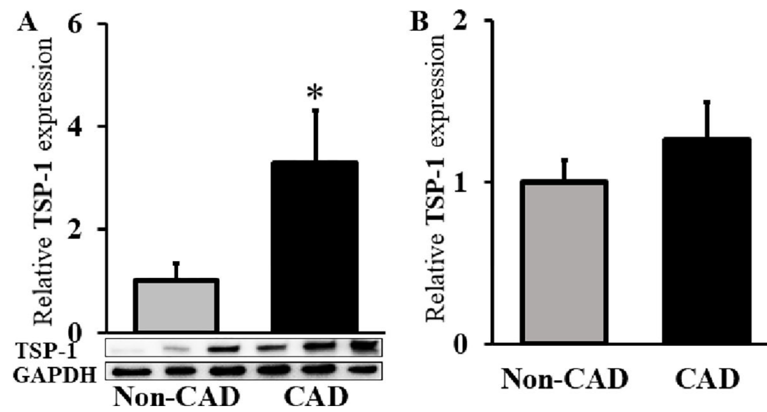

Figure 3: Comparison of TSP-1 protein expression in PAT and blood of CAD vs. non-CAD subjects.

a) PAT TSP-1 protein expression is significantly upregulated in subjects with CAD, quantified by Western immunoblot analysis and normalized to *GAPDH*. **b)** In a subset of individuals, exploratory analysis for plasma levels of TSP-1 did not demonstrate any significant differences, as quantified by ELISA (non-CAD, N=10; CAD, N=15). Data are expressed as relative fold-difference compared to non-CAD subjects indexed to 1. Data are displayed as mean ± SEM. * $p<0.05$.

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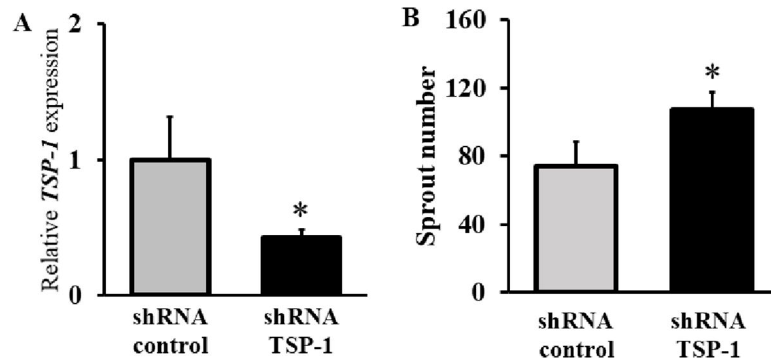

Figure 4: TSP-1 antagonism improves angiogenesis in CAD.

a) shRNA lentivirus induced a significant knock-down of TSP-1 gene expression in PAT. Data are expressed as relative fold-difference compared to control (sham) indexed to 1. **b)** TSP-1 silencing was associated with significant improvement in fat pad angiogenesis (n=5), compared to negative control lentivirus. Data are displayed as mean ± SEM. * $p<0.05$.

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Table 1.

Probes for qPCR

Gene Product	Assay ID
ANGPT1	Hs00181613_m1
Endostatin	Hs00181017_m1
FGF2	Hs00266645_m1
GAPDH	Hs02758991_g1
sFLT-1	Hs01052939_m1
TSP-1	Hs00962908_m1
VEGF-A	Hs00900055_m1

Table 2:

Clinical characteristics of the study population

Parameter	Total subjects (N=41)	Non-CAD (N=12)	CAD (N=29)	P value
Age, years	59.4±8	58.5±10	59.7±9	0.44
BMI, kg/m^2	32.7±6	35.0±9	31.8±4	0.19
Total cholesterol, mg/dl	170.8±48	171.4±39	170.6±52	0.45
LDL cholesterol, mg/dl	96.5±44	101±40	94.8±46	0.47
HDL cholesterol, mg/dl	45.0±12	51.1±16	42.7±10	0.05
Triglycerides, mg/dl	125.8±70	97.0±32	139.3±80	0.08
Hypertension, (%)	90	83	93	0.33
HbA1c, %	6.3±1.5	5.6±0.7	6.5±1.6	0.03
Diabetes mellitus, %	37	17	45	0.01
Creatinine, mg/dl	0.9±0.2	1.0±0.3	0.9±0.2	0.24
Left ventricular ejection fraction, %	52±14	62±6	49±15	<0.01

Data are mean ± SD. BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; and HbA1c, hemoglobin A1c

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