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Michael Tu April 13, 2023

Thrombospondin-1 as a Modulator of Carotid Plaque Formation and Arterial Remodeling in an Atherogenic, Disturbed-Flow Mouse Model

by

Michael Tu

Luke Brewster
Adviser

Biology

Luke Brewster

Adviser

Ken Moberg

Committee Member

Kate O'Toole

Committee Member

2023

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Michael Tu

Luke Brewster

Adviser

An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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By Michael Tu

Thrombospondin-1 (TSP-1) is a multifunctional extracellular glycoprotein that facilitates fibrosis through the activation of latent transforming growth factor-β (TGF-β). The TSP-1/TGF-β pathway contributes to arterial stiffening and atherosclerosis and is upregulated in response to factors such as increased blood glucose, disturbed blood flow, and oscillatory shear. This study aims to examine the role of TSP-1 on plaque formation and artery remodeling in an atherogenic TSP-1 knockout mouse model with disturbed carotid artery flow.

Both experimental (TSP-1 KO) and control (C57BL/6) groups underwent left-sided partial carotid ligation (PCL) at 12 weeks old. Atherogenic conditions were simulated by induction of hyperlipidemia via infection with a gain-of-function Pcsk9-AAV and initiation of a high-fat diet one week prior to PCL. Vessels were harvested 4 weeks after PCL, embedded in OCT and paraffin, frozen, and sectioned with thicknesses of 7-8 μm. Sections were stained, then imaged under a microscope. Quantification was performed in ImageJ.

Overall, results showed no significant differences between KO and control groups. However, male and female subgroup analyses showed male KO mice had a significantly smaller proportion of necrotic area (p=0.0024), and significantly larger inner and outer diameters (p=0.0023; p=0.0037) compared to controls. Female KO mice had significantly decreased lesion areas (p=0.0061) and significantly less CD68-positive percent area (p=0.0204) compared to the control. While these results support prior evidence that the absence of TSP-1 may improve plaque morphology, the differences interestingly varied between sexes.

Previous research in atherosclerosis supports our findings, although they have used different models of atherosclerosis. Research into genes and their expression that are associated with observed changes will be useful in fully mapping mechanisms, and understanding these pathways can influence the development of effective therapeutics.

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Thrombospondin-1 as a Modulator of Carotid Plaque Formation and Arterial Remodeling in an Atherogenic, Disturbed-Flow Mouse Model

Michael Tu[1], Luke Brewster MD, PhD, MA, RVT[2, 3]

1: Emory College of Arts and Sciences, 2: Emory School of Medicine, 3. Atlanta VA Medical Center

Plaque and remodeling modulated by TSP-1

Contact information: michael.z.tu2@gmail.com, lbrewst@emory.edu

Abstract

Thrombospondin-1 (TSP-1) is a multifunctional extracellular glycoprotein that facilitates fibrosis through the activation of latent transforming growth factor-□ (TGF-□). The TSP-1/TGF-□ pathway contributes to arterial stiffening and atherosclerosis and is upregulated in response to factors such as increased blood glucose, disturbed blood flow, and oscillatory shear. This study aims to examine the role of TSP-1 on plaque formation and artery remodeling in an atherogenic TSP-1 knockout mouse model with disturbed carotid artery flow.

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Overall, results showed no significant differences between KO and control groups. However, male and female subgroup analyses showed male KO mice had a significantly smaller proportion of necrotic area (p=0.0024), and significantly larger inner and outer diameters (p=0.0023; p=0.0037) compared to controls. Female KO mice had significantly decreased lesion areas (p=0.0061) and significantly less CD68-positive percent area (p=0.0204) compared to the control. While these results support prior evidence that the absence of TSP-1 may improve plaque morphology, the differences interestingly varied between sexes.

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with observed changes will be useful in fully mapping mechanisms, and understanding these pathways can influence the development of effective therapeutics.

Non-standard Abbreviations and Acronyms

AAV – adeno-associated virus

ApoE – Apolipoprotein E

D-flow – Disturbed Flow

ECM – Extracellular Matrix

IHC – Immunohistochemistry

KO – Knockout

LCA – Left Carotid Artery

LDL – Low-density Lipoprotein

OCT – Optimal Cutting Temperature compound

PCL – Partial Carotid Ligation

Pcsk9 – Proprotein convertase subtilisin/kexin 9

RCA – Right Carotid Artery

S-flow – Stable Flow

TGF-□ – Transforming Growth Factor Beta

TSP-1 – Thrombospondin-1

TSP-2 – Thrombospondin-2

WT – Wild-type

Introduction:

Thrombospondin-1 (TSP-1) is a glycoprotein that is part of the thrombospondin family. TSP-1 is a protean molecule, can bind to many ECM-associated proteins, and is involved in signaling pathways that are complex and contextual. Some notable receptor interactions of TSP-1 are with CD36 and CD45. CD36 is a receptor found on monocytes and capillary endothelial cells, among others. TSP-1–CD36 interaction is implicated in macrophage uptake of necrotic cells, activation of latent TGF-□, and anti-angiogenic effects (Chen et al.). The TSP-1–CD45/integrin-associated protein (IAP) interaction is involved in the inhibition of nitric oxide expression (Isenberg et al.). TSP-1 also has matricellular interactions, a notable one being the activation of TGF-□ by interaction with latent activating factor (LAF)/latency-associated peptide (LAP) (Murphy-Ullrich et al.). While TSP-1 interactions with matricellular proteins have been mapped out, their roles and contexts are not well understood (Resovi et al.). However, TSP-1 is associated with atherosclerosis, among other vascular conditions; previous research with arterial stiffening, a well-known determinant of cardiovascular health, indicated that TSP-1 had a role in stimulating profibrotic genes when endothelial cells were exposed to disturbed flow, and inhibiting TSP-1 resulted in less stiff arteries (Kim et al.).

While there is a clear association between TSP-1 and its role in arterial dysfunctions such as atherosclerosis, the mechanism(s) by which it acts is unknown. In leptin-driven atherosclerosis, knockout of TSP-1 resulted in improved metrics of vessel and atherosclerotic plaque measurements such as lipid accumulation, macrophage migration, and collagen deposition (Ganguly et al.). However, leptin appears to stimulate atherosclerosis progression by mechanisms different than those in shear stress and arterial blood flow. In the paper by Kim et

al., the reduction in arterial stiffening by knocking out TSP-1 was through the subsequent blocking of TGF-β (transforming growth factor-β) and its downstream activation of profibrotic genes. However, the blockage of TSP-1 and its more direct effects on ameliorating atherosclerosis have not been well-quantified.

This paper aims to uncover by which mechanisms TSP-1 may contribute to the development of flow-induced atherosclerosis and the effects that knocking out TSP-1 may have on said development. The goal of the paper is to use arteries from murine models to uncover the atherosclerotic mechanisms of TSP-1. We hypothesize that we will observe results similar to leptin-induced atherosclerotic mice where TSP-1 was knocked out, with improvements in plaque size, lipid deposition, and other metrics.

Methods:

Mice Husbandry:

Mice used in the experiments were C57BL/6 mice and C57BL/6 TSP-1$^{-/-}$ [Jackson Laboratory] and raised with littermates. Mice were initially fed regular chow. Animal husbandry practices were in accordance with Emory University IACUC protocols.

Vessel Harvesting:

Mice were asphyxiated with CO2 and sprayed with 70% ethanol. The heart was exposed by opening the thoracic cavity, and 0.5-1.0 cc of blood was collected with a sterile syringe from the right atrium or cavoatrial junction. After exsanguination, the right atrium was opened, and a needle was placed into the left ventricle for perfusion with 10-15 mL of cooled PBS performed over 10-15 minutes. Adequate perfusion was determined by blanching of major organs (liver, lungs). Aortic arches and carotid arteries were then harvested, imaged, and fixed in formalin. Vessels were then cut and embedded with OCT in 10 x 10 x 5 mm molds, then frozen in dry ice in preparation for section by cryostat.

Atherosclerosis Mice Model: Three Elements

Generation of a flow-based mouse model of atherosclerosis uses a partial carotid ligation. Nam et al. have shown that partial ligations resulted in a disturbed flow, defined as slow and oscillatory wall shear, which promotes inflammation in the artery of the mice. In their experiments, ligation of three of the four left caudal carotid arteries resulted in flow reversal during diastole in the LCA. Therefore, for these experiments, the first portion of our

atherosclerotic mice models included a partial left carotid ligation when the mice were twelve weeks old.

In addition, mice were infected with a gain-of-function mutant Pcsk9 in an adeno-associated virus a week before the partial ligation to accelerate the development of the atherosclerotic plaques. Pcsk9 is a protease with a role in the regulation of total cholesterol and low-density lipoproteins (LDL) and in research by (Maxwell and Breslow) the introduction of constitutively-active Pcsk9 through an adenovirus carrier resulted in significantly elevated total and LDL cholesterol. Finally, to greatly increase cholesterol and lipid levels, mice were fed a high-fat diet (**Table 1**). This triple combination of proatherogenic blood flow, inability to regulate blood cholesterol, and greater dietary cholesterol resulted in the formation of robust plaques in mice. Four weeks after partial carotid ligations, when the mice were sixteen weeks old, mice were euthanized and gross inspection of aortic arches (**Figure 1**) indicated that robust plaques had developed.

Plaque and Vessel Wall Analysis

To study plaque biology, mice arteries were embedded in paraffin for CD68 IHC staining and embedded in OCT for all other stains. For ideal samples, arteries were cut transversely in the middle of the plaque and the section is halved again. Arteries were cross-sectioned in sections of 8 um, and sections were then stained by various procedures, including hemotoxylin with eosin counterstain, Masson Trichome, Oil Red O, and IHC stains for CD68.

Stained slide images were taken and opened in ImageJ, an image-processing software. In ImageJ, quantifications for collagen composition, lipid percentage, CD68 expression, and necrotic area of the plaque were done. In addition, artery images were also quantified for the

circumference of the intima and the media. From the two circumferences, inner and outer diameters, as well as media thickness were calculated.

Statistical Tests

Statistical analyses were performed by using Graph-Pad Prism and Microsoft Excel statistical packages. For single comparisons, the paired or unpaired 2-tailed Student t-test, with significance set at $P<0.05$, was used (*$P<0.05$; †$P<0.01$; ‡$P<0.001$).

Results:

Plaque Biology:

Comparisons between WT mice and TSP-1 KO groups did not find any significant differences between the metrics used in **Figure 2**, such as lesion area (**2a**), lipid deposition (**2b**), collagen composition (**2c, 2d**), elastin breaks (**2e**), necrotic area (**2f**), and CD68-positive percent area (**2g**). However, when separating mice by sex and then looking at potential differences, there was a significant decrease in necrotic area in TSP-1 KO male mice which was not reflected in TSP-1 KO female mice (**Figure 3b**). On the other hand. TSP-1 KO female mice had a significant decrease in the lesion area (**Figure 4a**) and CD68-positive percent area (**Figure 4g**) which was not reflected in the male mice.

Arterial Remodeling:

Comparisons between WT mice and TSP-1 KO groups did not find any significant differences in media thickness (**Figure 2h**), and inner and outer diameter (**Figure 2i, 2j**). Segregation of mice by sex revealed that TSP-1 KO male mice had significant increases in both inner and outer diameter (**Figure 3i, 3j**).

Discussion

When looking at the biology of the plaque in our mice arteries, male KO mice had decreased necrotic percent area, whereas female KO mice had smaller plaques and less CD68-positive percent area. In addition, male KO mice had smaller inner and outer diameters than male WT mice. These differences indicate that inhibiting the action of TSP-1 has some effect on plaque morphology and arterial remodeling. Of interest are also differences in some metrics between male and female mice; looking at **Figures 3** and **4**, at graphs where male and female WT are about the same, we see trends where females have smaller lesions than males, whereas males have lower necrotic percent area and lipid deposition. Looking into these metrics, as well as sex-specific differences, and their correlated pathways may give further insight into the mechanisms of TSP-1.

However, our research does have limitations. Our model of atherosclerosis is an acute model of atherosclerosis; the combination of d-flow, Pcks9 transfection, and high-fat diet results in the formation of a robust plaque in 4 weeks post-ligation, while mice that have only been transfected with Pcsk9 and fed a high-fat diet develop plaques after 3 months (Bjørklund et al.). In addition, we did not conduct any pressure-diameter and pressure-compliance testing. However, the assumption is that results will be similar to past experiments comparing wild-type and TSP-1 KO mice. Finally, potential redundancies that may compensate for our global TSP-1 knockout were not considered. One gene that may compensate for TSP-1 deficiency is a related glycoprotein, thrombospondin-2 (TSP-2), which also has been shown to have anti-angiogenic properties, similar to TSP-1.

Our results with these differences in plaque biology between WT and KO mice have been seen in other papers, such as in Ganguly et al., referenced in the introduction. This paper aimed

to understand the role of TSP-1 in a hormone-driven model of atherosclerosis, with the hormone leptin. Their mice were either ApoE knockouts or double knockouts for ApoE and TSP-1. Mice were then either given daily injections of leptin. The results found significant differences between the two groups in metrics we also analyzed; TSP-1 deficiency prevented leptin-induced lipid burden, additional collagen deposition, lesion area increases, and less CD68-positive percent area, along with other changes. While some results were similar to ours, their model relied on leptin to drive the formation of plaques instead of d-flow. Past research in TSP-1 and the immune response in atherosclerotic plaques have found varying results as well. In a paper by Moura et al. studying the role of TSP-1 in plaque development, also with $ApoE^{-/-}$ and double knockout mice, when they stained plaques for CD45 at varying times (six months and nine months), they found a significant increase in CD45 percent area in the $TSP\text{-}1^{-/-}$ $ApoE^{-/-}$ group from six to nine months, while there was only an upward trend in the $ApoE^{-/-}$ group. Moura et al. interpreted this to indicate that TSP-1 provides a pathway by which the migration of immune cells is tempered. Our data differ as, at least in female mice, knocking out TSP-1 results in fewer CD68-expressing cells in the plaque. However, the goal and research methods by Moura et al. differ from ours. They focused more on how TSP-1 affects the rate of plaque development, using double knockout mice without any high-fat diet, ligation, or the use of hormones to promote plaque development. Their timeframe was also much different, looking at plaques at twelve months.

Arterial remodeling is also an aspect of atherosclerosis; research by Korshunov et al. on Glagov's phenomenon is a feature of arterial remodeling where in early stages of atherosclerosis (less than 40% stenosis), the arterial response is to increase the thickness of the vessel wall as well as the radius of the external elastic lamina, maintaining the vessel lumen area. However,

when percent stenosis exceeds a limit, the artery then remodels inwards, resulting in decreasing lumen area. Our data shows increases in inner and outer diameters in male TSP-1 KO mice, suggesting that male TSP-1 KO mice's carotid arteries undergo early arterial remodeling. However, to accurately determine this would require quantification of the lumen area over time.

Future directions include looking at if these results are consistent in other low-shear stress, oscillatory flow areas, such as the abdominal aorta or iliofemoral arteries. Another future direction is to look into sex-related differences in atherosclerosis and if and how these differences can change or alter plaque morphology or ethereal remodeling. In addition, while the data does indicate that certain metrics are different between the two groups, we do not have exact pathways through which these changes occur, requiring in vivo and in vitro testing of gene expression in TSP-1 KO cells/tissue to elucidate cellular mechanisms.

Our experiments looked at the effect that knocking out TSP-1 has on plaque formation and remodeling in flow-mediated atherosclerosis and have opened up new pathways to explore. Results were mostly consistent with previous research with other models of atherosclerosis. Although many of the compared metrics between the WT and TSP-1 KO mice were not significant, those that were, were all indicative of a more stable plaque and normal arterial remodeling, findings that can be useful in influencing future therapeutics.

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Disclosures:

I declare no conflicts of interest.

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Sex + Group	CHOL	TG	HDLc	Non-HDLc	LDLc
TSP1 Male	379.2 ± 191.24	68.6 ± 62.49	28.1 ± 20.55	351.1 ± 195.82	340.32 ± 188.06
TSP1 Female	385.8 ± 87.61	144.2 ± 172.17	37.3 ± 10.358	348.5 ± 91.04	319.66 ± 65.65
C57 Male	460.14 ± 47.13	337.29 ± 110.65	20.33 ± 11.38	439.81 ± 38.02	383.6 ± 23.62
C57 Female	416.71 ± 29.97	190.29 ± 149.24	14.81 ± 7.32	401.9 ± 23.52	370.19 ± 9.89

Table 1. Measurements of cholesterol and lipid levels in mice serum by sex and by groups. On average, all groups have elevated cholesterol levels compared to mice on a normal diet.

Figure 1. Aortic arch images with plaque development being the yellow-white enlarged areas of vessels. **a.** Male C57. **b.** Male TSP-1 KO. **c.** Female C57. **d.** Female TSP-1 KO.

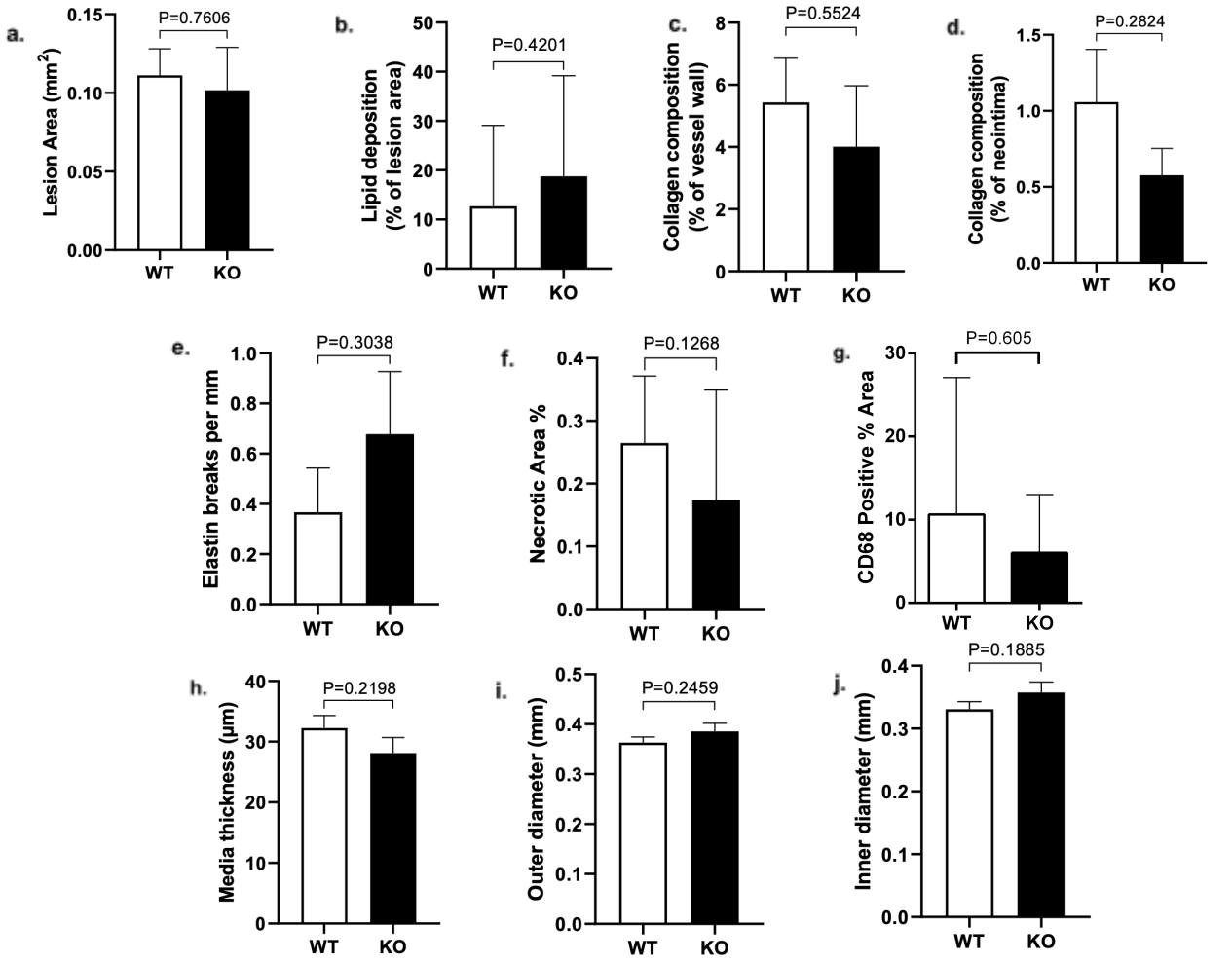

Figure 2. Data from WT and TSP-1 KO mice of both sexes. Graphs are metrics of plaque biology with **a.** lesion area, **b.** lipid deposition, **c** and **d.** collagen composition, **e.** elastin breaks, **f.** necrotic area, **g.** CD68 area and arterial remodeling with **h.** media thickness, and **i** and **j.** inner and outer diameter. No significant differences between the two groups in a Student's t-test. WT n=14, TSP-1 n=10. Mean ± SD.

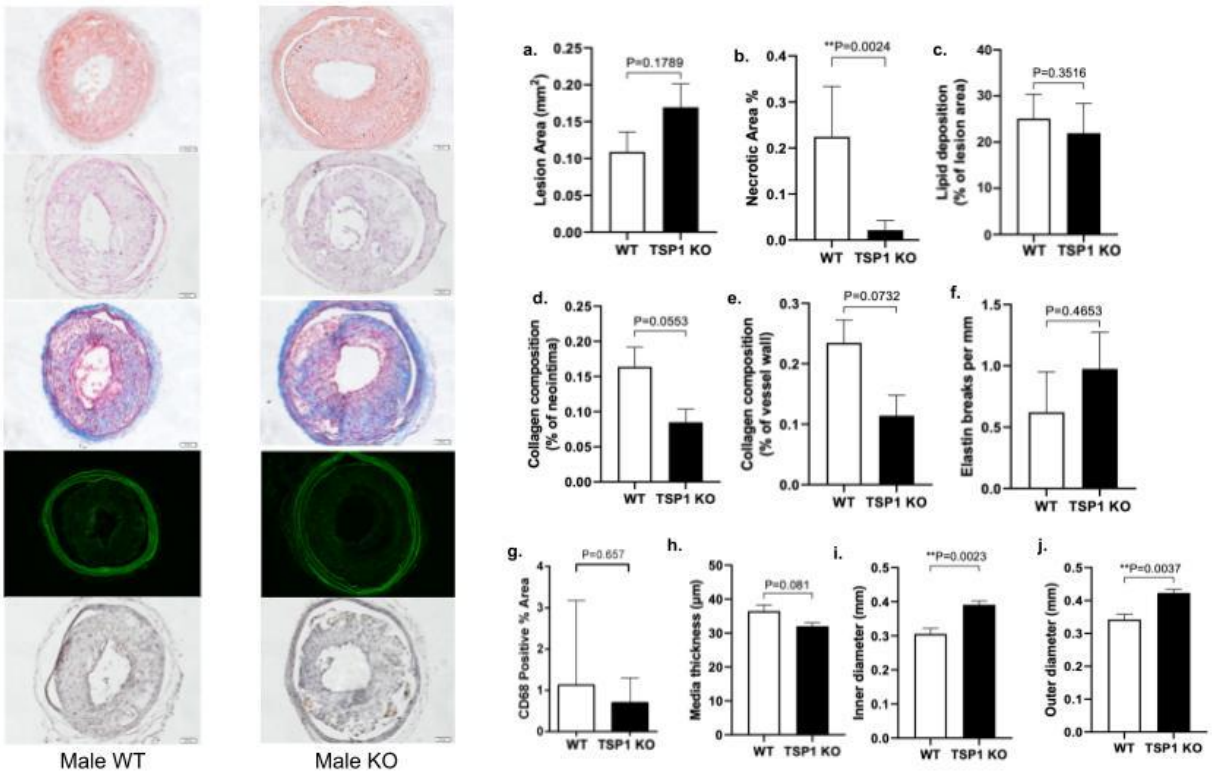

Figure 3. Representative images of male WT and KO sections. Stains from top to bottom are: Oil Red O, HE, Masson Trichrome, Elastin Autofluorescence, and CD68 IHC. Graphs comparing male WT and TSP-1 KO mice plaque characteristics and arterial remodeling. **a.** Lesion area, **b.** Necrotic area, **c.** Lipid deposition, **d** and **e.** Collagen composition, **f.** Elastin breaks, **g.** CD68 area, **h.** Media thickness, **i** and **j.** Diameters. WT n=7, KO n=5. Student's t-test. Mean ± SD.

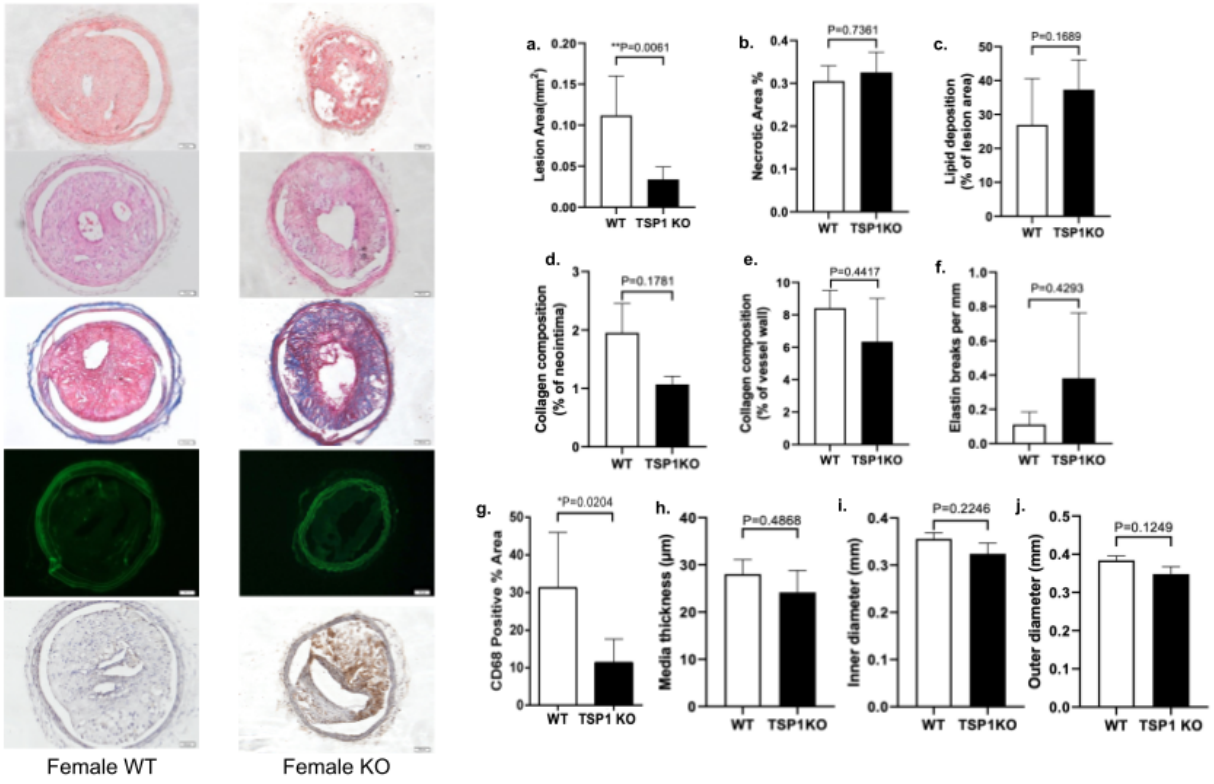

Figure 4. Representative images of female WT and KO sections. Stains from top to bottom are: Oil Red O, HE, Masson Trichrome, Elastin Autofluorescence, and CD68 IHC. Graphs comparing female WT and TSP-1 KO mice plaque characteristics and arterial remodeling. **a.** Lesion area, **b.** Necrotic area, **c.** Lipid deposition, **d** and **e.** Collagen composition, **f.** Elastin breaks, **g.** CD68 area, **h.** Media thickness, **i** and **j.** Diameters. WT n=7, KO n=5. Student's t-test. Mean ± SD.