SUPPLEMENTAL MATERIAL

Data S1. Systematic Search Protocol for most commonly used phenotypical and functional mesenchymal markers in the vascular research field[160,161]

Administrative information

Authors- contact and contributions:

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Contributions:

Both authors reviewed the titles, abstracts and full-texts for eligibility independently, based on the search strategy developed by author L.E. Bruijn in collaboration with search specialist J.W. Schoones, LUMC.

Financial support: none

Methods

Information sources: Pubmed and Embase

Pubmed search strategy:

(("Phenotype"[mesh:noexp] OR phenotyp*[tiab] OR "phenotypic modulation"[tw] OR "phenotypic regulation"[tw] OR "phenotypic differentiation"[tw] OR "phenotypic characterization"[tw] OR "phenotypic characterisation"[tw] OR "phenotypic diversity"[tw] OR "phenotypic heterogeneity"[tw] OR phenotypic modulat*[tw] OR

phenotypic regulat*[tw] OR phenotypic different*[tw] OR phenotypic character*[tw] OR phenotypic divers*[tw] OR phenotypic heterog*[tw] OR (("phenotypic"[ti] OR "pheno"[ti]) AND ("modulation"[ti] OR "regulation"[ti] OR "differentiation"[ti] OR "characterization"[ti] OR "characterisation"[ti] OR "diversity"[ti] OR "heterogeneity"[ti])) OR "vascular remodeling"[tw] OR "vascular remodelling"[tw]) AND ("Fibroblasts"[majr] OR "Myofibroblasts"[majr] OR "fibroblast"[ti] OR "fibroblasts"[ti] OR fibroblast*[ti] OR "myofibroblast"[ti] OR "myofibroblasts"[ti] OR myofibroblast*[ti] OR "Myocytes, Smooth Muscle"[majr] OR "smooth muscle cell"[ti] OR "smooth muscle cells"[ti]) AND ("Blood Vessels"[majr] OR "blood vessels"[ti] OR "blood vessel"[ti] OR "artery"[ti] OR "arteries"[ti] OR "aorta"[ti] OR "aortic"[ti] OR "Arteriosclerosis"[majr] OR "Atherosclerosis"[majr] OR atherosclero*[ti] OR arteriosclero*[ti] OR "coronary artery disease"[ti] OR "coronary artery diseases"[ti] OR "AAA"[ti] OR "Aortic Aneurysm, Abdominal"[majr] OR "Abdominal Aortic Aneurysm"[ti] OR "Abdominal Aortic Aneurysms"[ti]) NOT ("mesenchymal stem cells"[ti] OR "mesenchymal stem cell"[ti] OR "Mesenchymal Stromal Cells"[Majr]) AND ("1990/01/01"[PDAT] : "3000/12/31"[PDAT]) AND (english[la] OR dutch[la]))

Total number of references: 2698

Date: 9-12-2019

Data S2. Embase search strategy.

('Phenotype'/ OR Phenotyp*.ti,ab. OR 'Phenotypic modulation.mp. OR 'Phenotypic regulation'.mp. OR 'Phenotypic differentiation'.mp. OR 'Phenotypic characterization'.mp. OR 'Phenotypic characterisation'.mp. OR 'Phenotypic diversity'.mp. OR 'Phenotypic heterogeneity'.mp. OR Phenotypic modulat*.mp. OR Phenotypic regulat*.mp. OR Phenotypic different*.mp. OR Phenotypic character*.mp. OR Phenotypic divers*.mp. OR Phenotypic heterog*.mp. OR (('Phenotypic'.ti. OR 'Pheno'.ti.) AND ('Modulation'.ti. OR 'Regulation'.ti. OR 'Differentiation'.ti. OR 'Characterization'.ti. OR 'Characterisation'.ti. OR 'Diversity'.ti. OR 'Heterogeneity'.ti.)) OR 'Vascular Remodeling'.mp. OR 'Vascular Remodelling'.mp.) AND ('Fibroblasts'/ OR 'Myofibroblasts'/ OR 'Smooth Muscle cell'/ OR 'Fibroblast'.ti. OR 'Fibroblasts'.ti. OR Fibroblast*.ti. OR 'Myofibroblast'.ti. OR 'Myofibroblasts'.ti. OR Myofibroblast*.ti. OR 'Smooth muscle cell'.ti. OR 'Smooth muscle cells'.ti.) AND ('Blood Vessels'/ OR 'Arteriosclerosis'/ OR 'Atherosclerosis'/ OR 'Abdominal aortic aneurysm'/ OR 'Blood vessels'.ti. OR 'Blood vessel'.ti. OR 'artery'.ti. OR 'Arteries'.ti. OR 'Aorta'.ti. OR 'Aortic'.ti. OR Atherosclero*.ti. OR Arteriosclero*.ti. OR 'Coronary artery disease'.ti. OR 'Coronary artery diseases'.ti. OR 'AAA'.ti. OR 'Abdominal Aortic Aneurysm'.ti. OR 'Abdominal Aortic Aneurysms'.ti.) NOT 'Mesenchymal stroma cell'/ OR 'Mesenchymal stem cell'.ti. OR 'Mesenchymal stem cells'.ti.

Total number of references: 1371

Date: 10-12-2019

Data management: References were stored in a PDF-file.

Selection and data collection process: author L.E. Bruijn retrieved all included articles on 09-12-2019 in Pubmed and Embase with the search strategy. Gray literature was not located for this study. Next, the two reviewers independently undertook the initial selection based upon title, abstract and keywords. In case of disagreement, the two reviewers discussed whether the study should be included or excluded based on the initial selection. Subsequently, full texts were reviewed when eligibility was considered either definite or ambiguous.

Outcomes and prioritization:

Potential relevant differentiation/functional markers were extracted from the included studies for the qualitative synthesis. Studies were not assessed for quality as our research question was which mesenchymal and functional markers are most commonly used in the vascular research field and quality of the studies was therefore irrelevant.

Data S3. Movat Pentachrome Protocol.

Working solutions:

(A) 1% Alcian Blue Solution: 1 g Alcian Blue 8 GX (Merck, Burlington, US), 100 ml distilled water, 1 ml Glacial Acetic Acid (Sigma Aldrich, Saint Louis, US)

(B) Alkaline Alcohol solution: 10 ml Ammonium Hydroxide (Merck, Burlington, US), 90 ml Ethanol 100%.

(C) Elastic Hematoxylin Solution: 25 ml 10% Alcoholic Hematoxylin **(J)**, 25 ml Ethanol 100%, 25 ml 10% Ferric Chloride **(D)**, 25 ml Verhoeff's Iodine Solution **(K)**.

(D) 10% Ferric Chloride Solution: 10 g Ferric Chloride (Sigma Aldrich, Saint Louis, US), 100 ml distilled water

(E) 5% Sodium Thiosulfate Solution: 5g Sodium Thiosulfate (Sigma Aldrich, Saint Louis, US), 100 ml distilled water

(F) Biebrich Scarlet/Acid Fuchsin solution: pre-made from ScyTek Laboratories (Logan, United States).

(G) 1% Acetic Acid Solution: 1 ml Glacial Acetic Acid, 99 ml distilled water

(H) 5% Aqueous Phosphotungstic Acid solution: 5 g Phosphotungstic Acid (Sigma Aldrich, Saint Louis, US), 100 ml distilled water

(I) 4% Alcoholic Saffron Solution: 4 g Saffron (Safranor Safran du Gâtinais, Échilleuses, France), 100 ml Ethanol 100%.

(J) 10% Alcoholic Hematoxylin Solution: 10 g Hematoxylin (Merck, Burlington, US), 100 ml Ethanol 100%

(K) Verhoeff's Iodine Solution: 2 g Iodine Crystals (Sigma Aldrich, Saint Louis, US), 4 g Potassium Iodide (Sigma Aldrich, Saint Louis, US), 100 ml distilled water

Protocol:

1. Deparaffinization and rehydration of slides. 2. Rinse slides in distilled water. 3. Stain in 2 changes with **(A)** , both times for 15-25 minutes. 4. Rinse slides in running warm to hot water until clear. 5. Place slides in **(B)** for 30 minutes, then rinse in running tap water. 6. Stain in **(C)** for 20 minutes. 7. Rinse in running warm tap water. 8. Differentiate in 2% aqueous **(D)** for 5 seconds-2 minutes. 9. Place slides in **(E)** for about 1 minute. 10. Wash in running tap water and rinse in distilled water. 11. Stain in **(F)** for 1-1.5 minutes. 12. Rinse in distilled water. 13. Rinse in **(G)** for 7-12 seconds. 14. Place slides in **(H)** for 7-12 minutes. 15. Rinse in distilled water. 16. Rinse in **(G)** for 8-10 seconds. 17. Place in 2 changes of Ethanol 100%. 18. Stain in **(I)** for 1.5 minute and quickly rinse in Ethanol 100%. 19. Dehydration of slides.

Table S1. Excluded full-texts in Systematic Search.

Number	Publication	Reason
1	Owens GK. Molecular control of vascular smooth muscle cell differentiation and phenotypic plasticity. *Novartis Found Symp.* 2007;283:174-91.	Conference proceeding.
2	Bentzon JF, Majesky MW. Lineage tracking of origin and fate of smooth muscle cells in atherosclerosis. *Cardiovasc Res*. 2018;114(4):492-500.	No marker proteins for differentiation described.
3	Maegdefessel L, Rayner KJ, Leeper NJ. MicroRNA regulation of vascular smooth muscle function and phenotype: early career committee contribution. *Arterioscler Thromb Vasc Biol.* 2015;35(1):2-6.	No marker proteins for differentiation described.
4	Matchkov VV, Kudryavtseva O, Aalkjaer C. Intracellular Ca^{2+} signalling and phenotype of vascular smooth muscle cells. *Basic Clin Pharmacol Toxicol*. 2012;110(1):42-8.	No marker proteins for differentiation described.
5	Song Z, Li G. Role of specific microRNAs in regulation of vascular smooth muscle cell differentiation and the response to injury. *J Cardiovasc Transl Res*. 2010;3(3):246-50.	No marker proteins for differentiation described.
6	Erlinge D. Extracellular ATP: a central player in the regulation of vascular smooth muscle phenotype. Focus on "Dual role of PKA in phenotype modulation of vascular smooth muscle cells by extracellular ATP". *Am J Physiol Cell Physiol.* 2004;287(2):C260-2.	No marker proteins for differentiation described.
7	Bochaton-Piallat ML, Bäck M. Novel concepts for the role of smooth muscle cells in vascular disease: towards a new smooth muscle cell classification. Cardiovasc Res. 2018;114(4):477-480.	No marker proteins for differentiation described.
8	Shi N, Mei X, Chen SY. Smooth Muscle Cells in Vascular Remodeling. *Arterioscler. Thromb. Vasc. Biol*. 2019;39(12):e247-e252.	No marker proteins for differentiation described.
9	Basatemur GL, Jørgensen HF, Clarke MCH, Bennett MR, Mallat Z. Vascular smooth muscle cells in atherosclerosis. *Nat Rev Cardiol.* 2019;16(12):727-744.	No full-text available in Leiden University Medical Library collection.
10	Kannenberg F, Gorzelniak K, Jager K, Fobker M, Rust S, Repa J, Roth M, Bjorkhem I, Walter M. Characterization of cholesterol homeostasis in telomerase immortalized tangier disease fibroblasts reveals marked phenotype variability. *J. Biol. Chem.* 2013;288(52):36936-36947.	No marker proteins for differentiation described.

Table S2. Excluded phenotypical markers identified with Systematic Search.

Protein marker	Reason for exclusion
DDR2[15,310]	<3 references
NGS[97]	Primarily pericyte marker
Anti-reticular fibroblast marker (RFM)[97]	<3 references
MHC class II[302]	<3 references
CD4[302]	<3 references
Acting binding proteins Cofilin[16] Profilin[16] Talin[176]	<3 references
MYPT-1[16]	<3 references
Cytoskeleton proteins Tubulin[98] Metavinculin[40,42,111]	<3 references Vinculin selected
Leiomodin-1[111]	<3 references
Integrins (A1,A7,B1)[211,294]	<3 references
Myocardin[211,222,294]	Master regulator of SMC differentiation. Panel of contractile markers included yet.
N-Cadherin/T-Cadherin[86,294]	<3 references
Calmodulin[111,214,249]	Telokin selected
LPP[38]	<3 references
Myosin light chains (LC17a en LC17b)[39]	<3 references
Cysteine- and glycine-rich protein 1[91]	<3 references
1E12[226]	<3 references
Phospholamban[228,300]	Expressed specifically in cardiac muscle.
Aquaporin-1[228,300]	<3 references
Osteoglycin[228]	<3 references
Ubiquitin[228]	<3 references
APEG-1[294]	<3 references
CRP-2[294]	<3 references
MAS5[300]	<3 references

Table S3. Excluded full-texts in synthetic/inflammatory marker search.

Number	Publication	Reason
1	Owens GK. Molecular control of vascular smooth muscle cell differentiation and phenotypic plasticity. *Novartis Found Symp*. 2007;283:174-91.	Conference proceeding.
2	Bentzon JF, Majesky MW. Lineage tracking of origin and fate of smooth muscle cells in atherosclerosis. *Cardiovasc Res.* 2018;114(4):492-500.	No marker proteins for differentiation described.
3	Maegdefessel L, Rayner KJ, Leeper NJ. MicroRNA regulation of vascular smooth muscle function and phenotype: early career committee contribution. *Arterioscler Thromb Vasc Biol*. 2015 Jan;35(1):2-6.	No marker proteins for differentiation described.
4	Matchkov VV, Kudryavtseva O, Aalkjaer C. Intracellular Ca^{2+} signalling and phenotype of vascular smooth muscle cells. *Basic Clin Pharmacol Toxicol.* 2012;110(1):42-8.	No marker proteins for differentiation described.
5	Song Z, Li G. Role of specific microRNAs in regulation of vascular smooth muscle cell differentiation and the response to injury. *J Cardiovasc Transl Res*. 2010;3(3):246-50.	No marker proteins for differentiation described.
6	Erlinge D. Extracellular ATP: a central player in the regulation of vascular smooth muscle phenotype. Focus on "Dual role of PKA in phenotype modulation of vascular smooth muscle cells by extracellular ATP". *Am J Physiol Cell Physiol.* 2004;287(2):C260-2.	No marker proteins for differentiation described.
7	Bochaton-Piallat ML, Bäck M. Novel concepts for the role of smooth muscle cells in vascular disease: towards a new smooth muscle cell classification. *Cardiovasc Res.* 2018;114(4):477-480.	No marker proteins for differentiation described.
8	Shi N., Mei X., Chen S.-Y. Smooth Muscle Cells in Vascular Remodeling. *Arterioscler. Thromb. Vasc. Biol.* 2019;39(12):e247-e252.	No marker proteins for differentiation described.
9	Basatemur GL, Jørgensen HF, Clarke MCH, Bennett MR, Mallat Z. Vascular smooth muscle cells in atherosclerosis. *Nat Rev Cardiol.* 2019;16(12):727-744.	No full-text available in Leiden University Medical Library collection.
10	Reddy MA, Villeneuve LM, Wang M, Lanting L, Natarajan R. Role of the lysine-specific demethylase 1 in the proinflammatory phenotype of vascular smooth muscle cells of diabetic mice. *Circ Res*. 2008;103(6):615-23. Retraction in: *Circ Res.* 2009;105(6):e9.	Retracted publication.
11	Ramos KS, Weber TJ, Liau G. Altered protein secretion and extracellular matrix deposition is associated with the proliferative phenotype induced by allylamine in aortic smooth muscle cells. *Biochem J.* 1993;289.	No protein markers for differentiation or proliferative/ synthetic features described.
12	5. Stengel D, O'Neil C, Brochériou I, Karabina SA, Durand H, Caplice NM, Pickering JG, Ninio E. PAF-receptor is preferentially expressed in a distinct synthetic phenotype of smooth muscle cells cloned from human internal thoracic artery: functional implications in cell migration. *Biochem Biophys Res Commun*.2006;346(3):693-9.	No protein markers for differentiation or proliferative/ synthetic features described.
13	Liu L, Abramowitz J, Askari A, Allen JC. Role of caveolae in ouabain-induced proliferation of cultured vascular smooth muscle cells of the synthetic phenotype. *Am J Physiol Heart Circ Physiol.* 2004;287(5):H2173-82.	No protein markers for differentiation or proliferative/ synthetic features described.
14	Rybalkin SD, Bornfeldt KE, Sonnenburg WK, Rybalkina IG, Kwak KS, Hanson K, Krebs EG, Beavo JA. Calmodulin-stimulated cyclic nucleotide phosphodiesterase (PDE1C) is induced in human	No protein markers for differentiation or proliferative/

	arterial smooth muscle cells of the synthetic, proliferative phenotype. *J Clin Invest*. 1997;100(10):2611-21.	synthetic features described.
15	Wang Y, Lindstedt KA, Kovanen PT. Phagocytosis of mast cell granule remnant-bound LDL by smooth muscle cells of synthetic phenotype: a scavenger receptor-mediated process that effectively stimulates cytoplasmic cholesteryl ester synthesis. *J Lipid Res.* 1996;37(10):2155-66.	No protein markers for differentiation or proliferative/ synthetic features described.
16	Kannenberg F, Gorzelniak K, Jager K, Fobker M, Rust S, Repa J, Roth M, Bjorkhem I, Walter M. Characterization of cholesterol homeostasis in telomerase immortalized tangier disease fibroblasts reveals marked phenotype variability. *J. Biol. Chem. 2013*;288(52):36936-36947	No marker proteins for differentiation described.

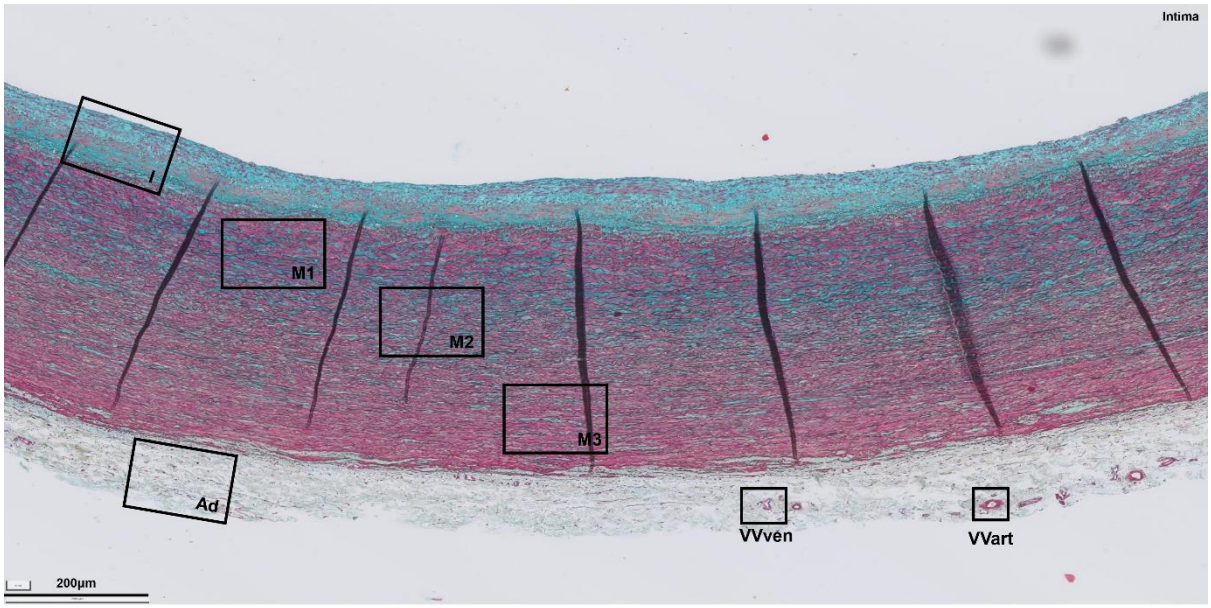
Table S4. Excluded synthetic IHC markers.

Marker	Reason for exclusion
(pro-)Collagen II, III, VIII[40,208,268]	Among all the collagen subtypes, type I collagen is considered to be most abundant in vascular fibrous pathology.
Collagenase IV[294]	<3 references
Heat shock protein 47 (Hsp47)[195]	<3 references
ECM components Chondroitin sulfate[98] Vitronectin[40] Tenascin[232] Tropoelastin[232] Fibrillin[233] Osteoglycin[235] Syndecan-1/4[104,294] Versican[20] Aortic-carboxypeptidase-like protein (ALCP)[42,91]	<3 references
Matrix Gla Protein (MGP)[232,235,317]	Predominantly extracellular protein
TGN46[278]	<3 references
Intermediate filaments GFAP[278] Cytokeratin 8 and 18[111,232]	<3 references
Cyclophilin A[281]	<3 references
Zona occludens 2- protein[111]	<3 references
Cingulin[111]	<3 references
uPa[111]	<3 references
tPA[111]	<3 references
Connexin 43[104,194]	<3 references
IGF-BP2[102]	<3 references
TSP-1[40]	<3 references
Cytochrome P-45OIAI[232]	<3 references
Osteoprotegerin[241]	<3 references
SMPD3[105]	<3 references
Sortilin-1[105]	<3 references

Table S5. Excluded pro-inflammatory IHC markers.

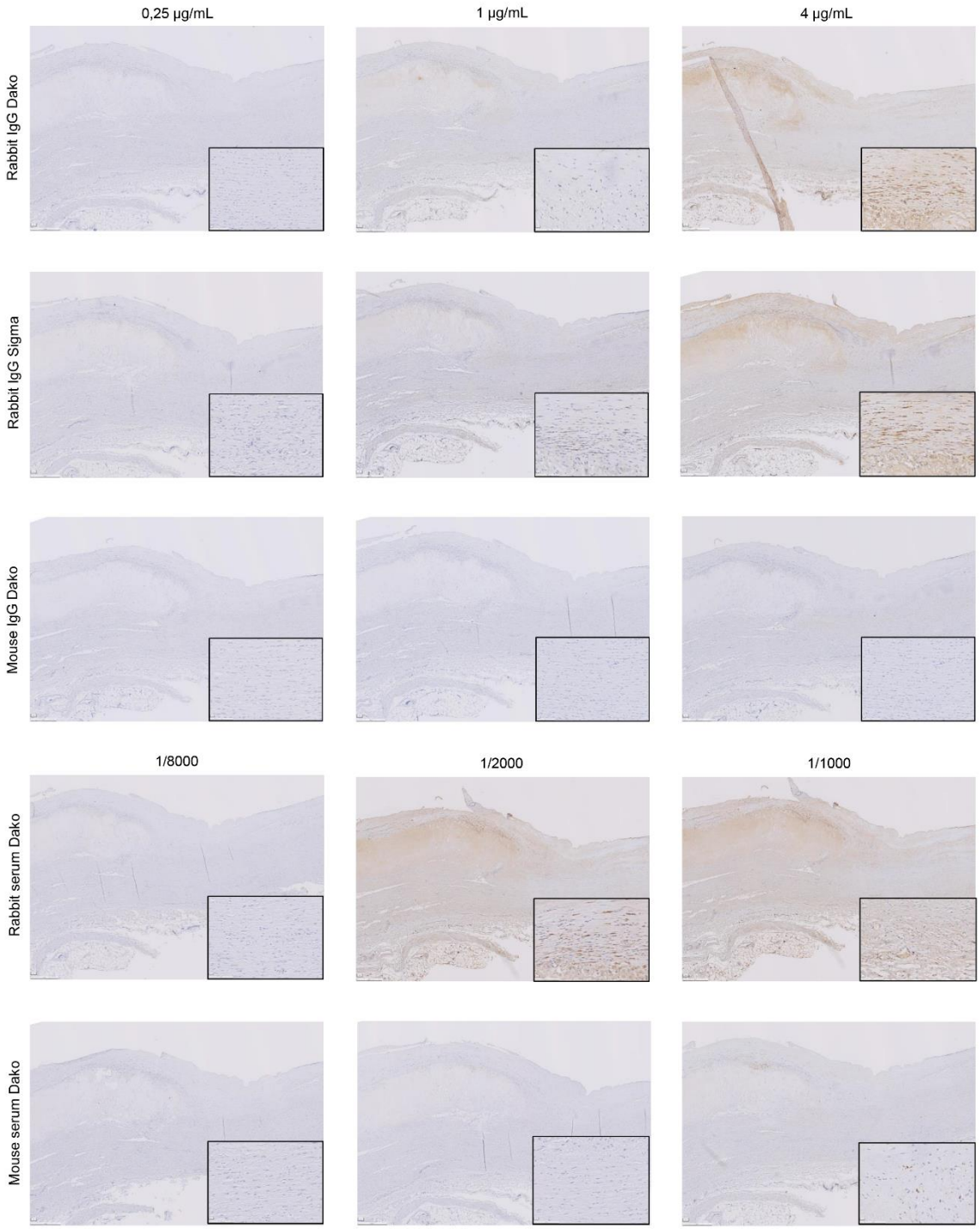
Marker	Reason for exclusion
IL-1a[259,263]	<3 references
IL-1β[106,112,162]	Not detectable at protein level[294]
TNF-α[106,112,162]	Very low expression at protein level[294]
MCSF[109]	<3 references
TGF-β[177]	<3 references
Cell activation markers (ICAM-1, VCAM-1, E-selectin, MMP-2, MMP-3, MMP-7, MMP-9)[29,106,112,177,203,260,318]	NF-κB selected as central regulator
CCR2[19]	<3 references
MHCII[14]	<3 references
CXCL1[107,239]	<3 references

Figure S1. Defining the regions of interest within atherosclerotic lesions for semi-quantitative scoring system.



Abbreviations: I. Intima **M1.** Inner media zone **M2.** Middle media zone **M3.** Outer media zone **Ad.** Adventitia **VVven.** Venule-like vasa vasora **VVart.** Arteriole-like vasa vasora.

Figure S2. Rabbit IgG in concentrations higher than 1 µm/mL produce significant background.



Overview of rabbit isotype controls in several concentrations (on early fibroatheroma (EFA); 2,5x and 26x magnification). Non-specific binding of rabbit IgG is significantly increased in rabbit IgG concentrations of 1 µg/mL or higher. This phenomenon was not observed in mouse IgG or mouse sera.

Figure S3. Disqualification of Thy-1/CD90 and FSP-1/S100A4 as universal mesenchymal lineage markers.

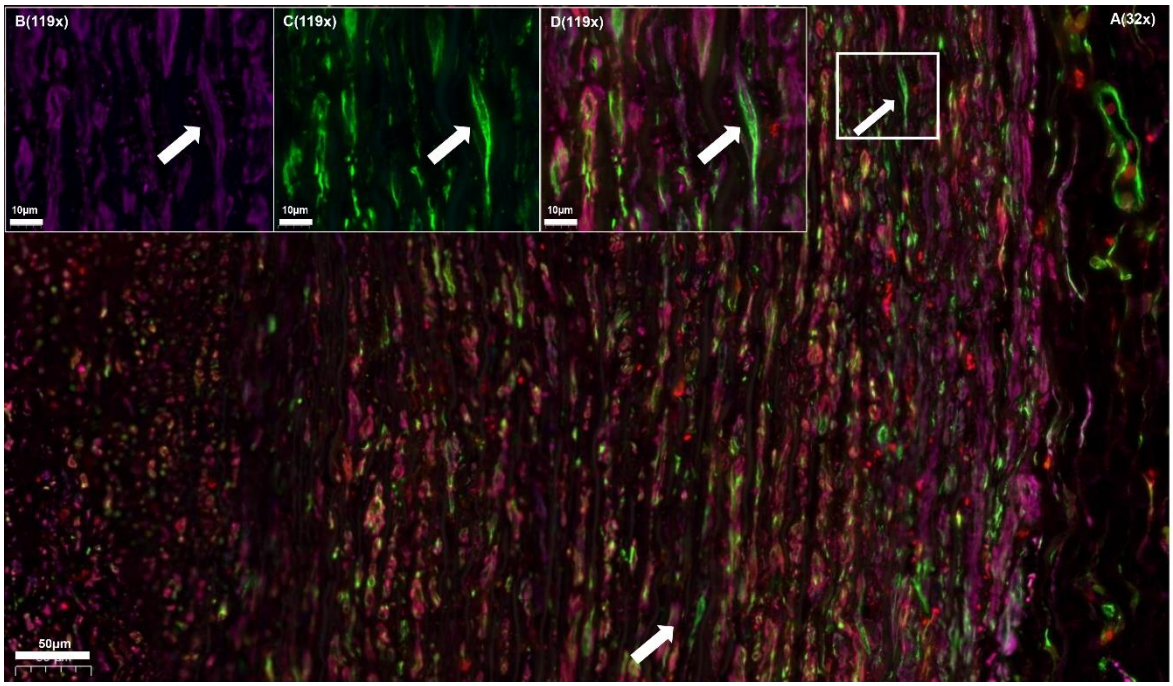


Fig S3.1. A. The majority of spindle shaped cells in the media of AIT are triple positive for Vimentin^{+} (in *green*; AF488; FITC-channel), FSP-1^{+} (in *red*; AF546; TRITC-channel) and αSMA^{+} (in *magenta*; AF647; Cy5-channel). The inserts show single channel signals for αSMA^{+} (**B**) and Vimentin^{+} (**C**). Insert (**D**) shows the presence of αSMA^{+}/Vimentin^{+}/FSP-1^{-} double positive subpopulation.

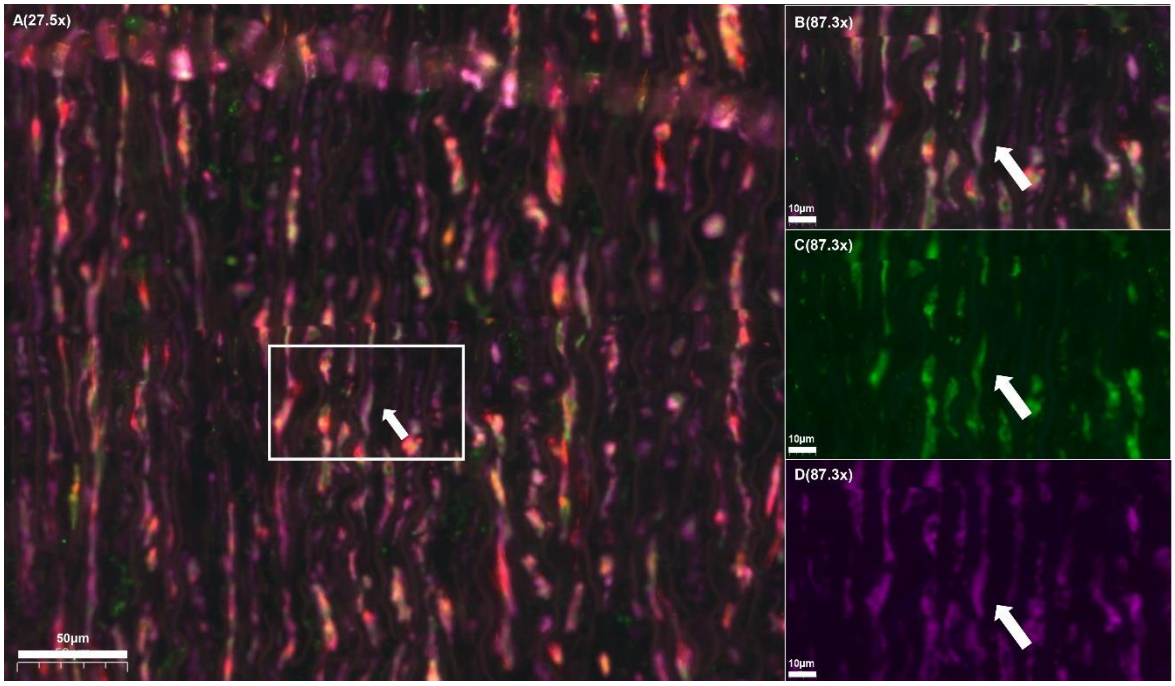


Fig S3.2. A. The majority of spindle shaped cells in the media of AIT are triple positive for Vimentin^{+} (in *green*; AF488; FITC-channel), CD90^{+} (in *red*; AF546; TRITC-channel) and αSMA^{+} (in *magenta*; AF647; Cy5-channel). The inserts show single channel signals for Vimentin^{+} (**C**) and αSMA^{+} (**D**). Insert (**B**) shows the presence of αSMA^{+}/Vimentin^{+}/CD90^{-} double positive subpopulation.

Figure S4. Challenging Vimentin as an all-inclusive panmesenchymal marker.

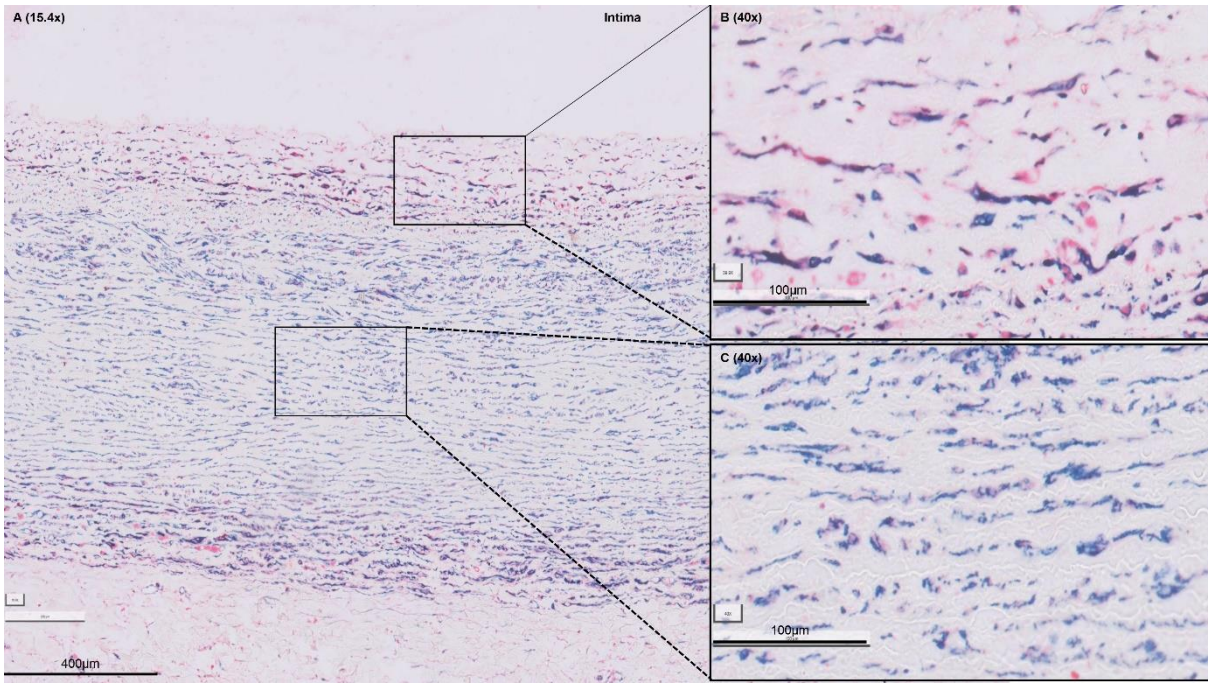


Figure S4.1: A. Adaptive Intimal Thickening (AIT) section double IHC stained for FAP (in *red*) and Vimentin (in *blue*). In AIT, FAP and Vimentin show almost complete (**B**/**C**; in *purple*).

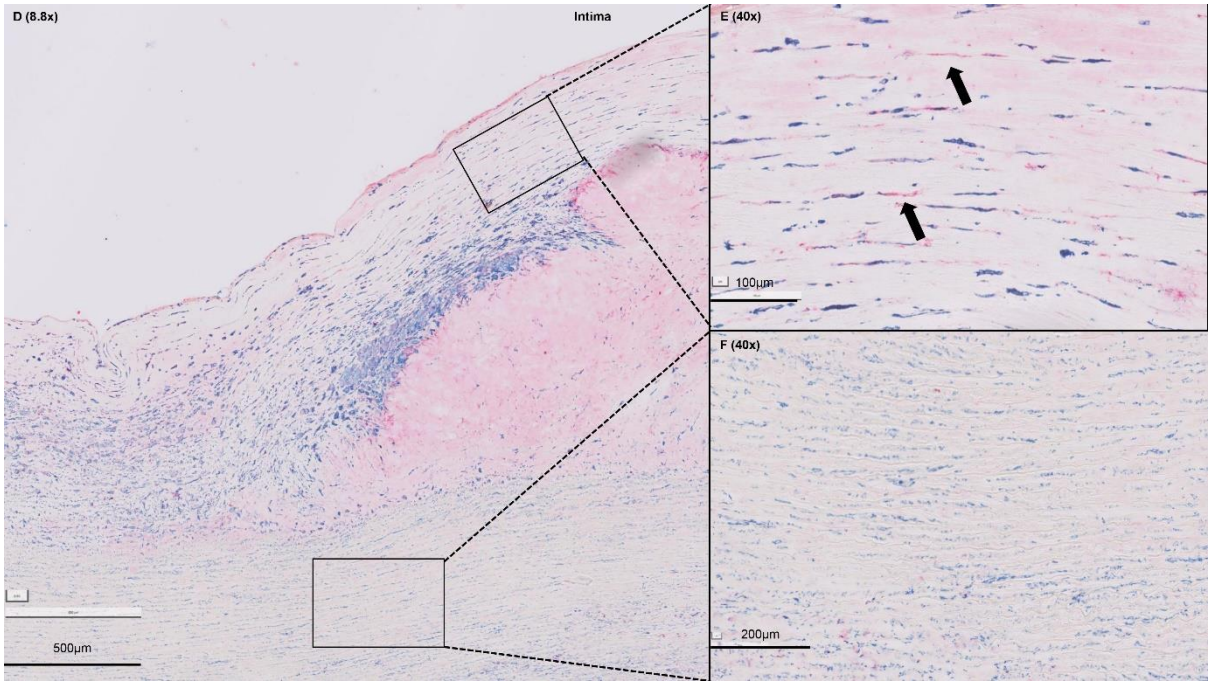


Figure S4.2. A. Late Fibroatheroma (LFA) section double IHC stained for FAP (in *red*) and Vimentin (in *blue*). During atherogenic progression, although FAP expression remained stable in the medial zones (**F**), FAP expression is unstable in the cap region, shown by spindle-shaped single FAP^{+} cells (**E; arrows,** *in red*), challenging Vimentin as an all-inclusive panmesenchymal marker.

Figure S5. Double Vimentin^{+}/αSMA^{+} cells in the cap of progressive atherosclerotic lesions (LFA and TCFA) and stabilized atherosclerotic lesions (HR).

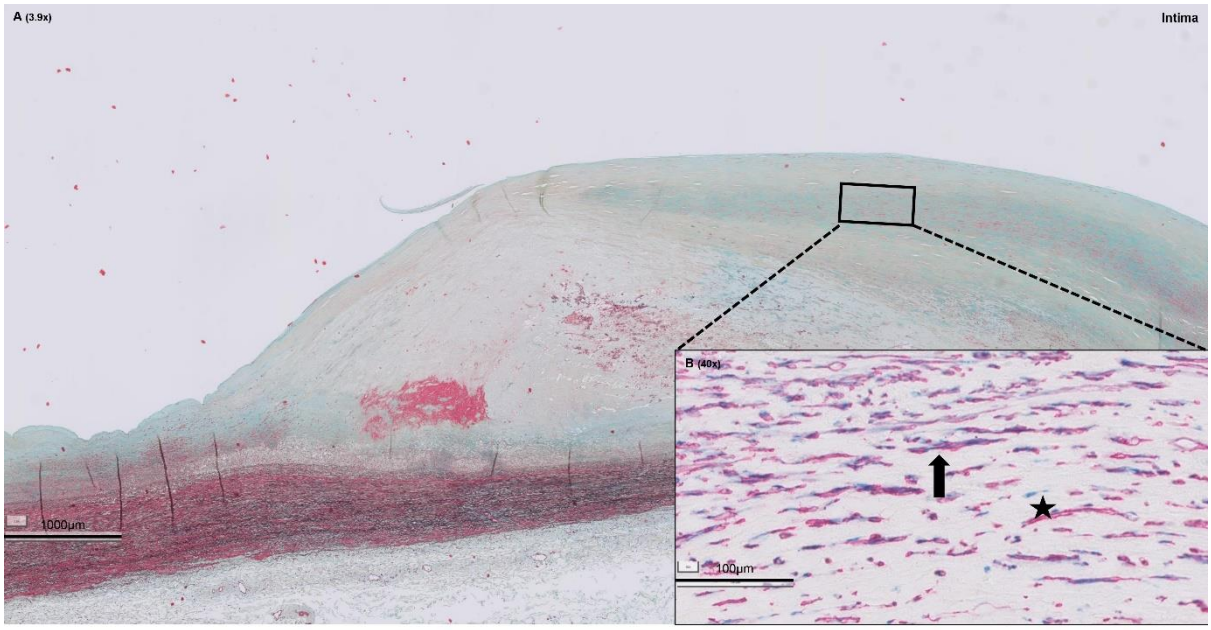


Figure S5.1: A. Movat Pentachrome staining of LFA (Late Fibroatheroma).

Legend: *red*, smooth muscle cells/fibrin; *violet*: leukocytes; *black*: elastin; *blue*: proteoglycans/mucins; *yellow*: collagen. Various shades of green reflect colocalization of collagen (*yellow*) and proteoglycans (*blue*).

B. Insert of the cap double IHC stained for Vimentin (in *blue*) and αSMA (in *red*), showing that the cap is mesenchymal cell (Vimentin+) rich. Approximately 80% of the Vimentin^{+} cells were double Vimentin^{+}/αSMA^{+} (**arrow in B**, in *purple*; star indicates a single Vimentin^{+}/αSMA^{-} cell).

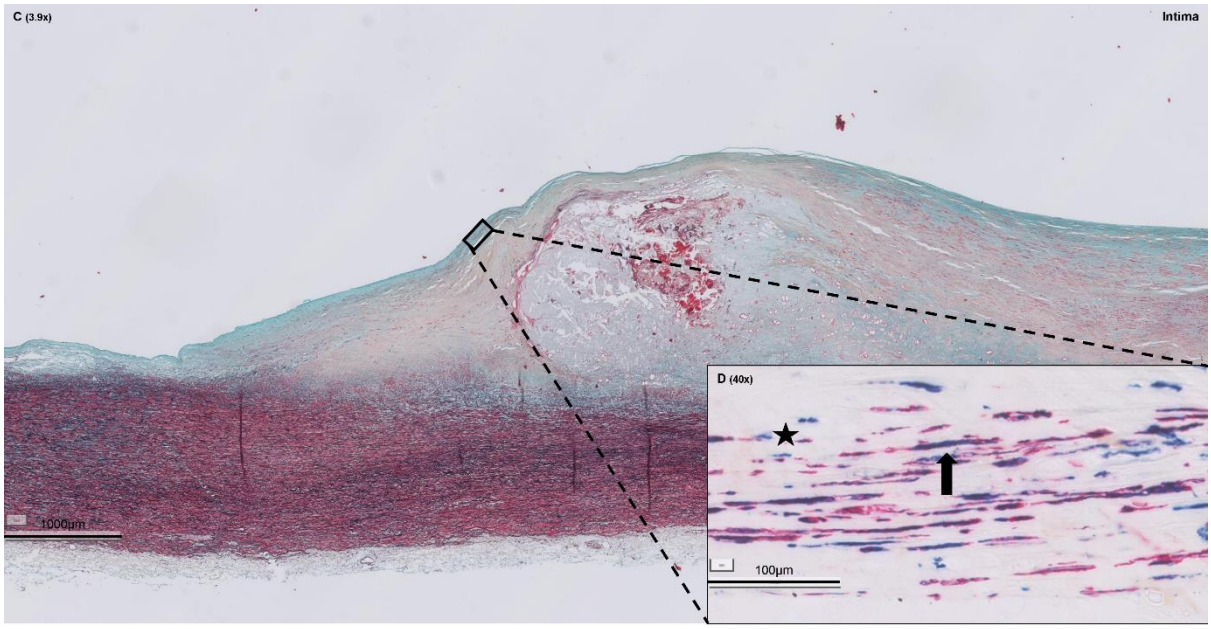


Figure S5.2: C. Movat Pentachrome staining of TCFA (Thin cap fibroatheroma). For legend see Figure S5.1.

D. Insert of the cap double IHC stained for Vimentin (in *blue*) and αSMA (in *red*). Transition from a LFA to a TCFA was associated with a clear decrease in cell density. Approximately 80% of the Vimentin^{+} cells were double Vimentin^{+}/αSMA^{+} (**arrow in D**, in *purple*; star indicates a single Vimentin^{+}/αSMA^{-} cell).

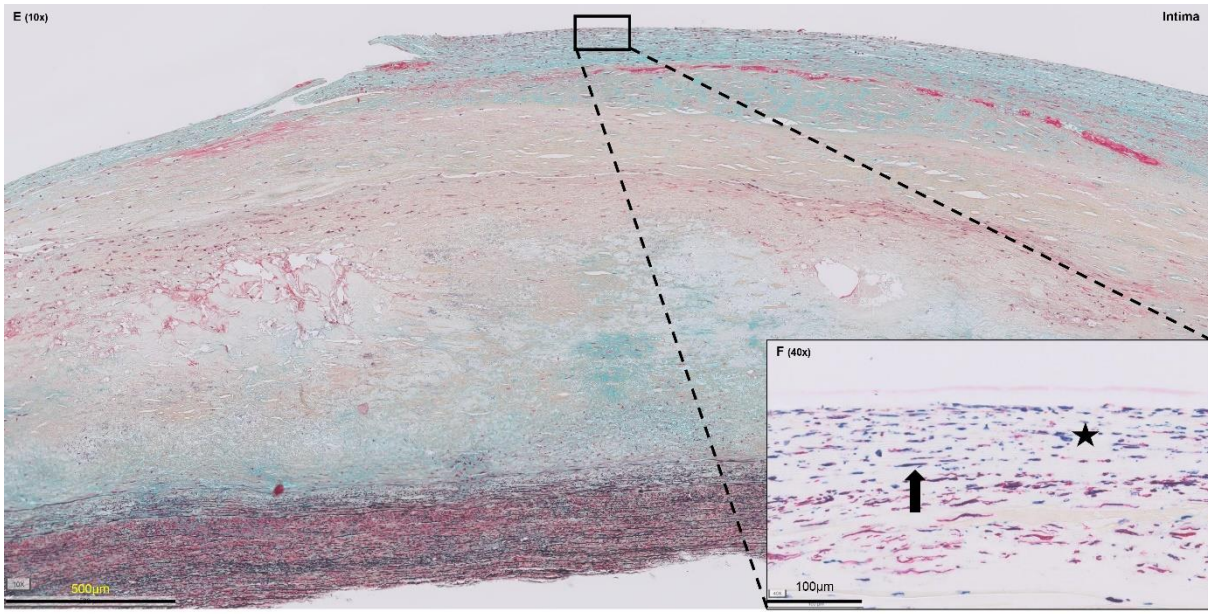


Figure S5.3: E. Movat Pentachrome staining of HR (Thin cap fibroatheroma). For legend see Figure S5.1.

F. Within the cell-rich/proteoglycan-rich luminal granulation tissue that is associated with healing of a ruptured atherosclerotic lesions (HR), approximately 80% of the Vimentin^{+} cells (in *blue*) were double Vimentin^{+}/αSMA^{+} (**arrow in D**, in *purple*; star indicates a single Vimentin^{+}/αSMA^{-} cell).

Figure S6. Vimentin, Thy-1/CD90, S100A4/FSP-1 and FAP are limited in mesenchymal lineage specificity: expression in monocytic cells.

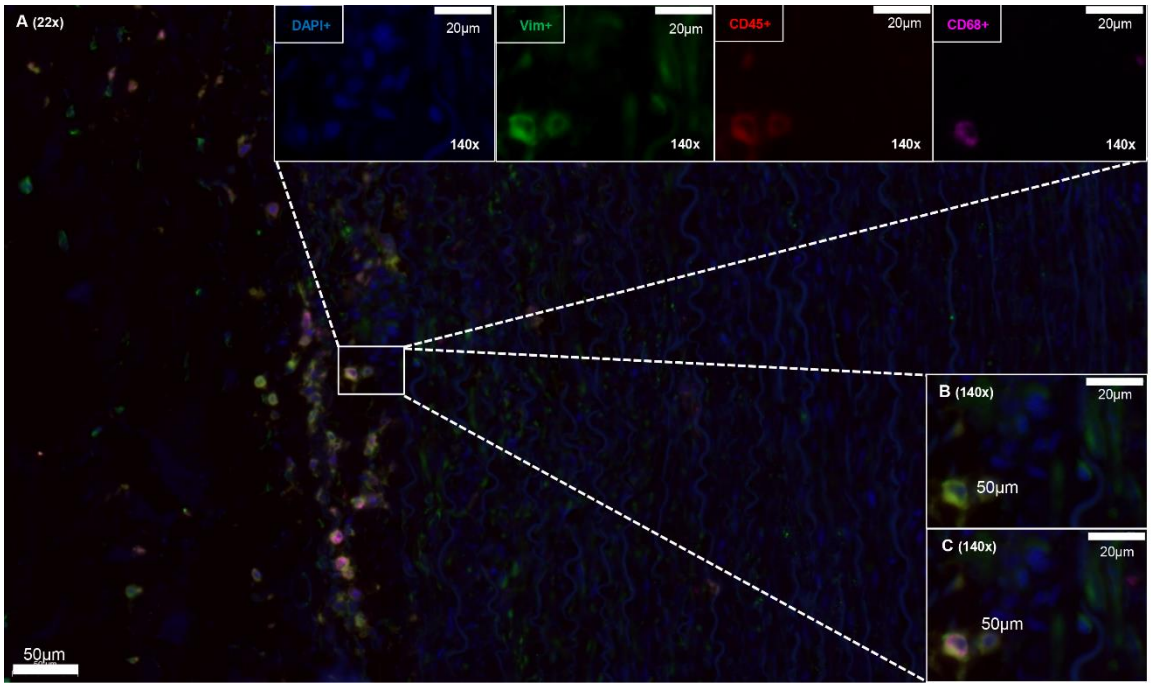


Figure S6.1: A. Triple IF staining of Vimentin (Vim), clone 1A4, (in *green*; AF488; FITC-channel), CD45 (in *red*; Vulcan Red; TRITC-channel) and CD68 (in *magenta*; AF647; Cy5-channel) in the adventitia. Nuclei are DAPI-stained (*blue*). Arterial elastic laminae in the medial layer are occasionally visible in greenblue due to auto-fluorescence. The four top inserts show the single channel information for Dapi, Vimentin, CD45 and CD68 (from left to right). Inserts on the down right show overlay information for **(B)** $CD45^+$/$Vimentin^+$ cells, respectively of which a part is $CD68^+$/$Vimentin^+$/$CD45^+$**(C)**.

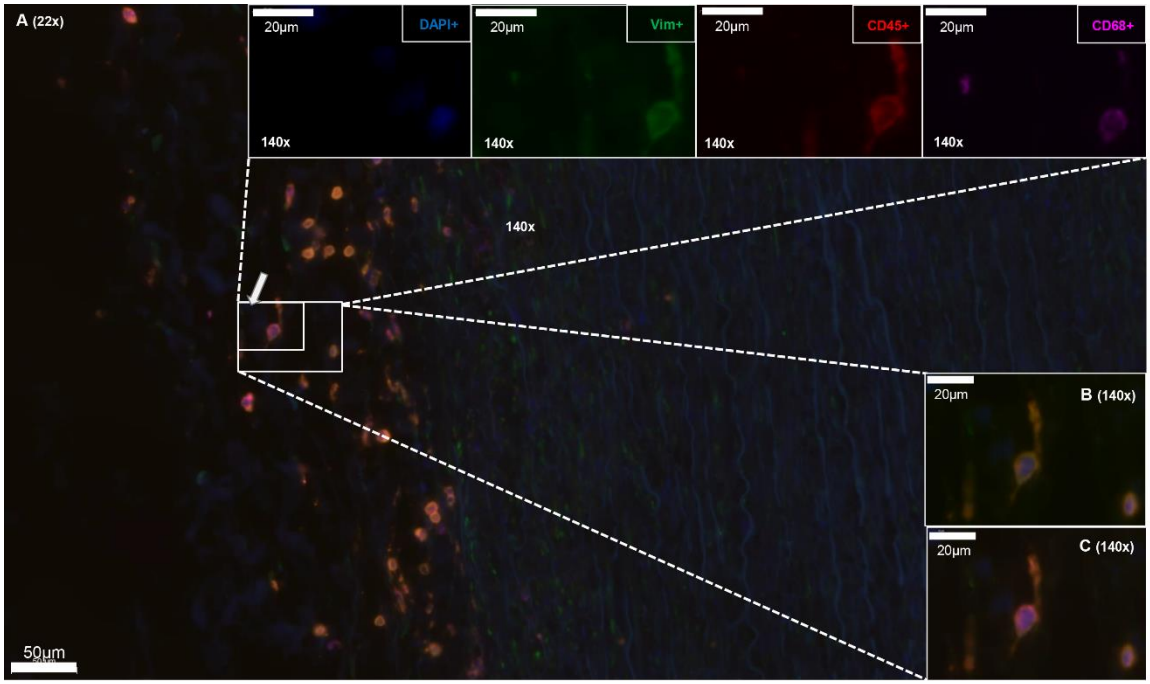
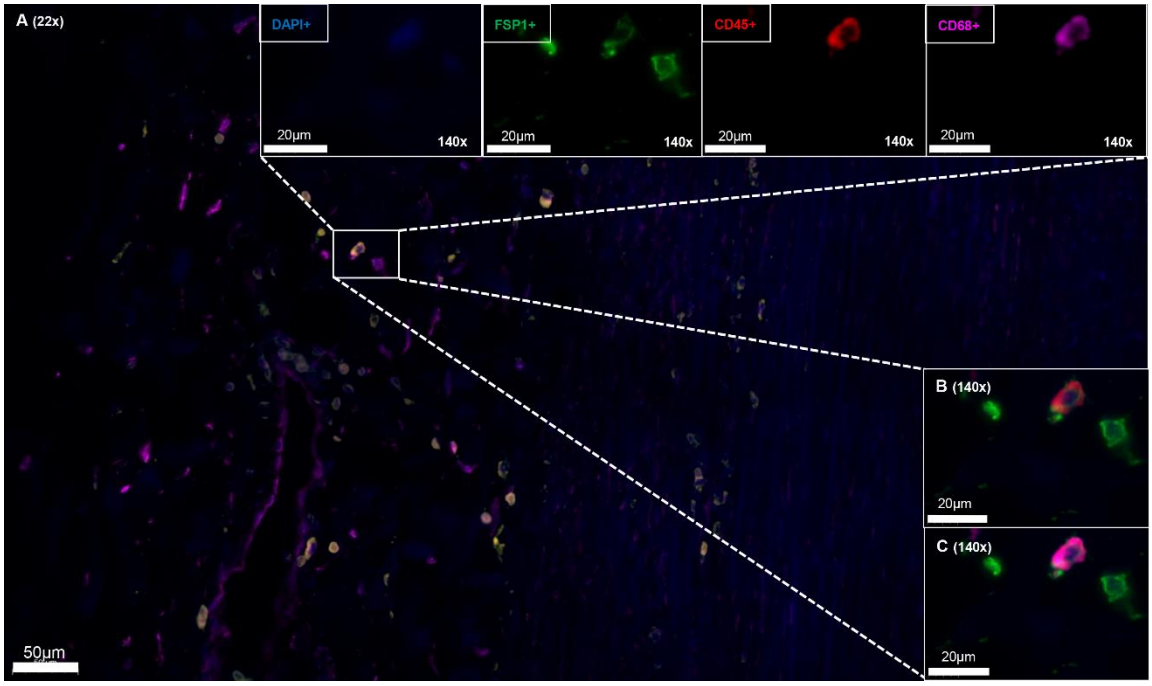


Figure S6.2: A. Triple IF staining of Vimentin (Vim), clone VI-10, (in *green*; AF488; FITC-channel), CD45 (in *red*; Vulcan Red; TRITC-channel) and CD68 (in *magenta*; AF647; Cy5-channel) in the adventitia. Nuclei are DAPI-stained (*blue*). Arterial elastic laminae in the medial layer are occasionally visible in greenblue due to auto-fluorescence. The four top inserts show the single channel information for Dapi, Vimentin, CD45 and CD68 (from left to right). Inserts on the down right show overlay information for **(B)** $CD45^{+}$/$Vimentin^{+}$ cells, respectively of which a part is $CD68^{+}$/$Vimentin^{+}$/$CD45^{+}$**(C)**.



FigureS6.3: A. Triple IF staining of FSP-1 (in *green*; AF647; Cy5-channel), CD45 (in *red*; Vulcan Red; TRITC-channel) and CD68 (in *magenta*; AF488; FITC-channel) in the adventitia. Nuclei are DAPI-stained (*blue*). Arterial elastic laminae in the medial layer are occasionally visible in greenblue due to auto-fluorescence. The four top inserts show the single channel information for Dapi, FSP-1, CD45 and CD68 (from left to right). Inserts on the down right show overlay information for **(B)** $CD45^{+}/FSP\text{-}1^{+}$ cells, respectively of which a part is $CD68^{+}/FSP\text{-}1^{+}/CD45^{+}$**(C)**.

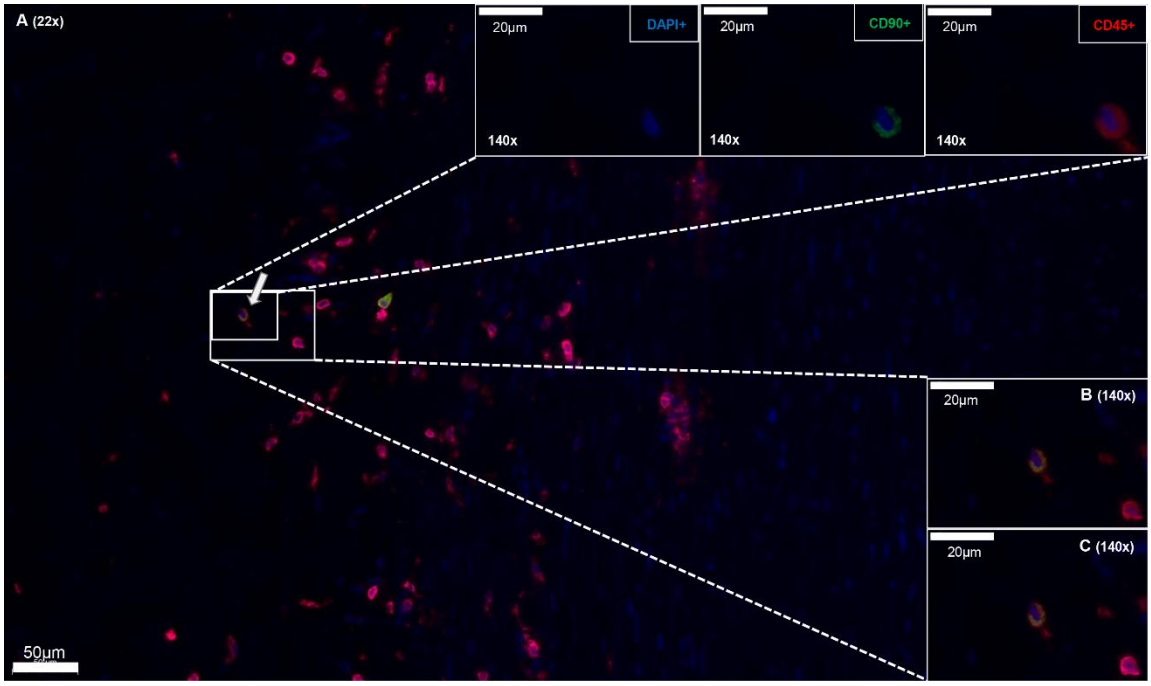


Figure S6.4: A. Triple IF staining of CD90 (in *green*; AF488; FITC-channel), CD45 (in *red*; Vulcan Red; TRITC-channel) and CD68 (in *magenta*; AF647; Cy5-channel) in the adventitia. Nuclei are DAPI-stained (*blue*). Arterial elastic laminae in the medial layer are occasionally visible in greenblue due to auto-fluorescence. The four top inserts show the single channel information for Dapi, CD90, CD45 and CD68 (from left to right). Inserts on the down right show overlay information for **(B)** $CD45^+/CD90^+$ cells, respectively of which a part is $CD68^+/CD90^+/CD45^+$**(C)**.

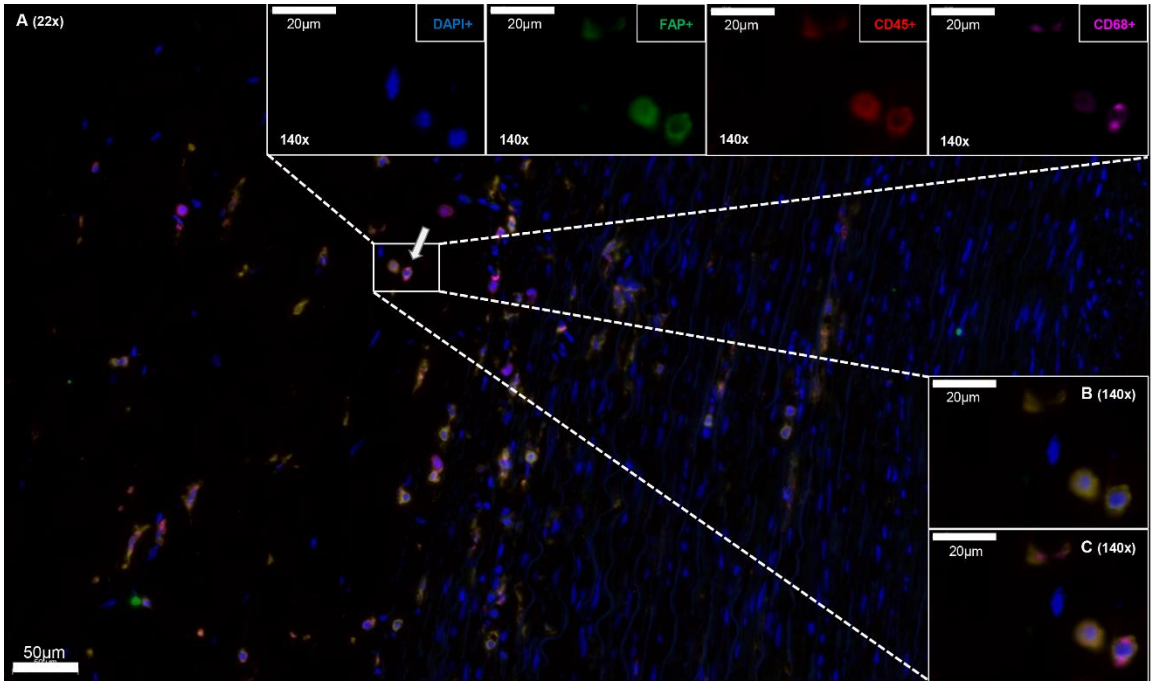


Figure S6.5: A. Triple IF staining of FAP (in *green*; AF488; FITC-channel), CD45 (in *red*; Vulcan Red; TRITC-channel) and CD68 (in *magenta*; AF647; Cy5-channel) in the adventitia. Nuclei are DAPI-stained (*blue*). Arterial elastic laminae in the medial layer are occasionally visible in greenblue due to auto-fluorescence. **(B)** The four top inserts show the single channel information for Dapi, FAP, CD45 and CD68 (from left to right). Inserts on the down right show overlay information for **(B)** $CD45^{+}/FAP^{+}$ cells, respectively of which a part is $CD68^{+}/FAP^{+}/CD45^{+}$**(C)**.

Figure S7. Limited mesenchymal lineage specificity of Vimentin: expression in endothelial cells. Thy-1/CD90, S100A4/FSP-1 and FAP are not expressed in endothelial cells.

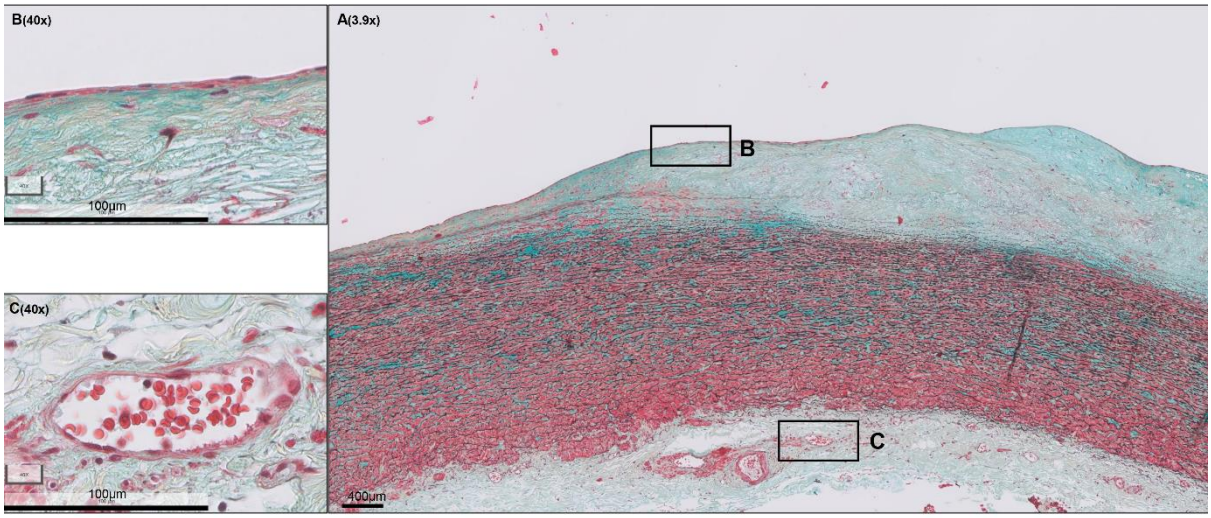


Figure S7.1: **A**. Movat Pentachrome staining of EFA (Early Fibroatheroma). Legend: *red*, smooth muscle cells/fibrin; *violet*: leukocytes; *black*: elastin; *blue*: proteoglycans/mucins; *yellow*: collagen. Various shades of green reflect colocalization of collagen (*yellow*) and proteoglycans (*blue*). **B**. Close up of intact endothelium in the arterial wall and of endothelial cells in a vasum vasorum (**C**).

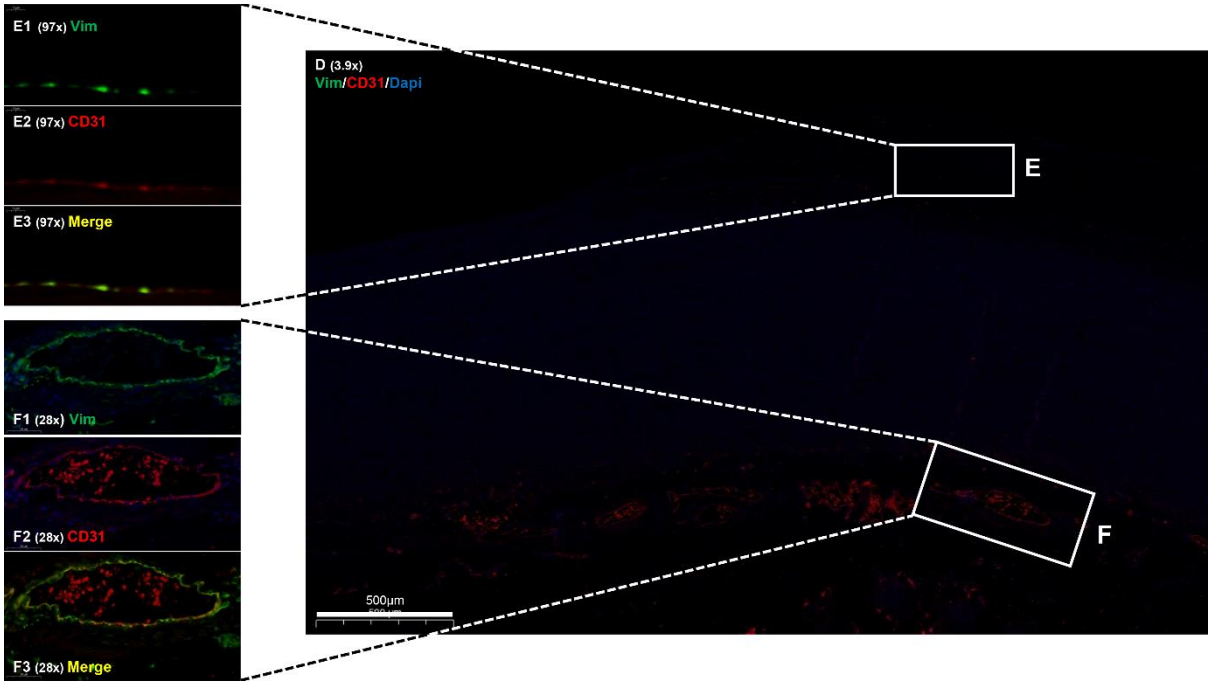


Figure S7.2: D. Double IF staining of Vimentin (cytoplasma staining, in *green*; AF488; FITC-channel) and CD31 (plasma membrane and cell junction staining, in *red*; Vulcan Red; TRITC-channel) of a consecutive section of the EFA shown in **A**. Nuclei are DAPI-stained (in *blue*).

The inserts on the left shown single channel information for Vimentin (**E1/F1**) and CD31 (**E2/F2**). Vimentin and CD31 colocalize in the arterial intima (**E3**) and in endothelial cells in the vasa vasora (**F3**).

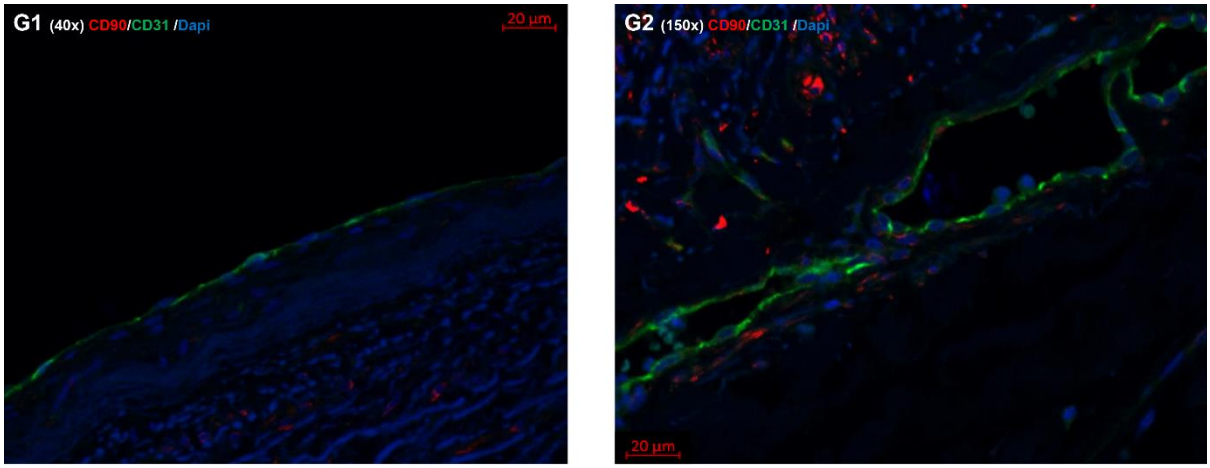


Figure S7.3: **G1**. Close up of single positive $CD31^+$ (in *green*; AF488; FITC-channel; plasma membrane and cell junction staining)/$CD90^-$ endothelial cells in the arterial wall. **G2**. Close up of $CD90^+/CD31^-$ cells (in *red*; Vulcan Red; TRITC-channel; plasma membrane staining) that are in close proximity of $CD31^+$ endothelial cells (in *green*; AF488; FITC-channel; plasma membrane and cell junction staining) in vasa vasora. Nuclei are DAPI-stained (in *blue*).

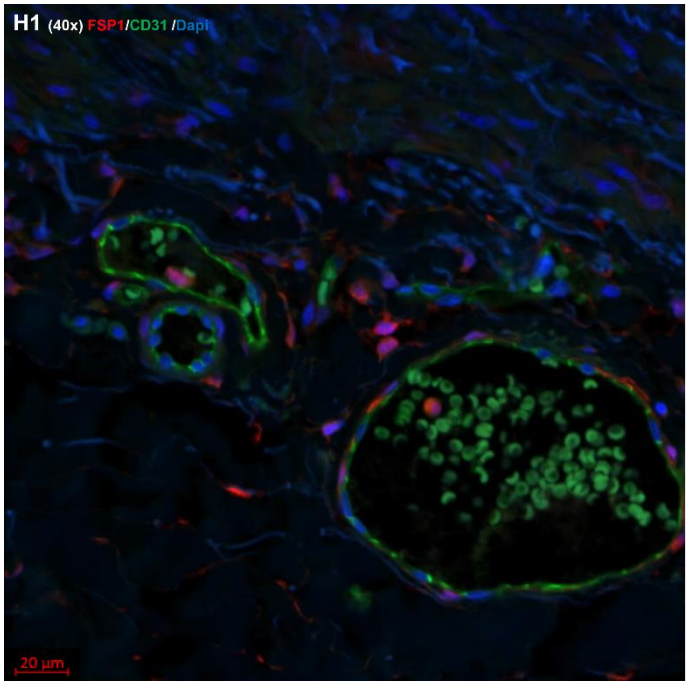


Figure S7.4: H1. Close up of FSP-1^{+} cells (in *red*; Vulcan Red; TRITC-channel, nucleus and cytoplasm staining) are in close proximity of single CD31^{+} endothelial cells (in *green*; AF488; FITC-channel; plasma membrane and cell junction staining) in vasa vasora. Nuclei are DAPI-stained (in *blue*).

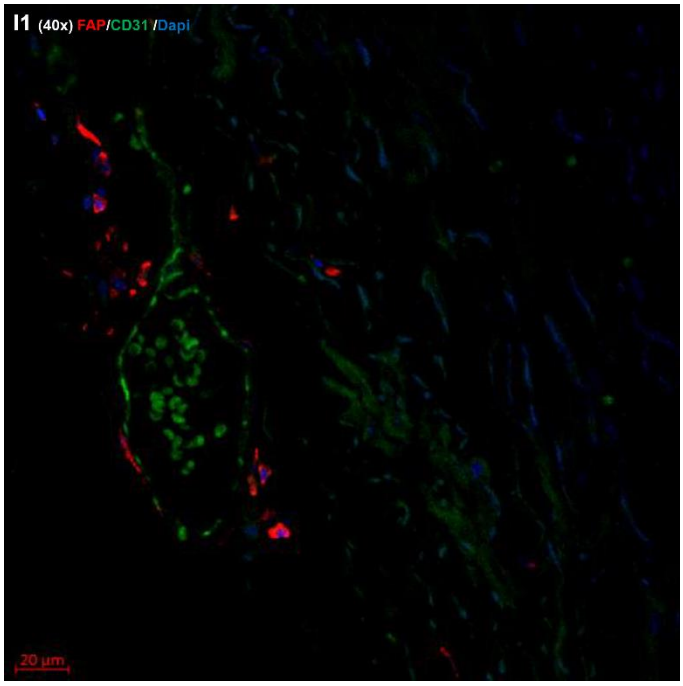


Figure S7.5: H1. Close up of single FAP$^+$ cells (in *red*; Vulcan Red; TRITC-channel, nucleus and cytoplasm staining) that are in close proximity of CD31$^+$ endothelial cells (in *green*; AF488; FITC-channel; plasma membrane and cell junction staining) in vasa vasora. Nuclei are DAPI-stained (in *blue*).

Figure S8. Role for EndoMT/ stem cells in vascular pathology.

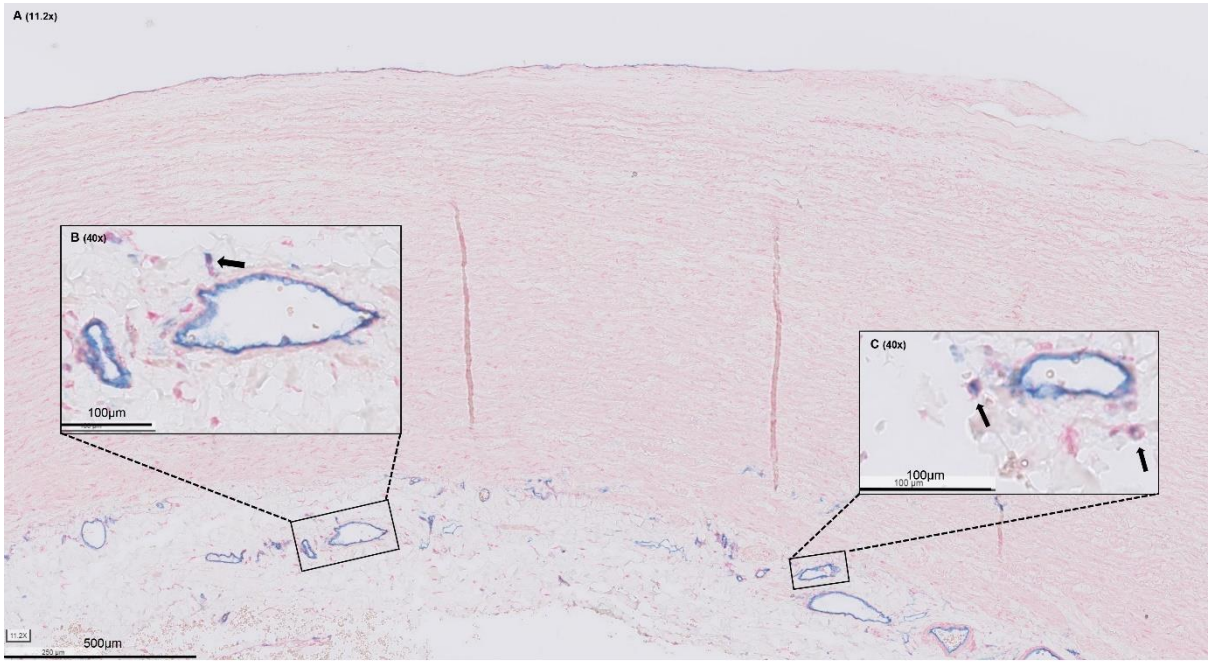


Figure S8.1: A. Double IHC staining of CD31 (in *blue*) and Vimentin (in *red*) on LFA. The inserts (**B/C**) show solitary double Vimentin^{+}/CD31^{+} cells in the vicinity of the vasa vasora (in purple; **arrows**).

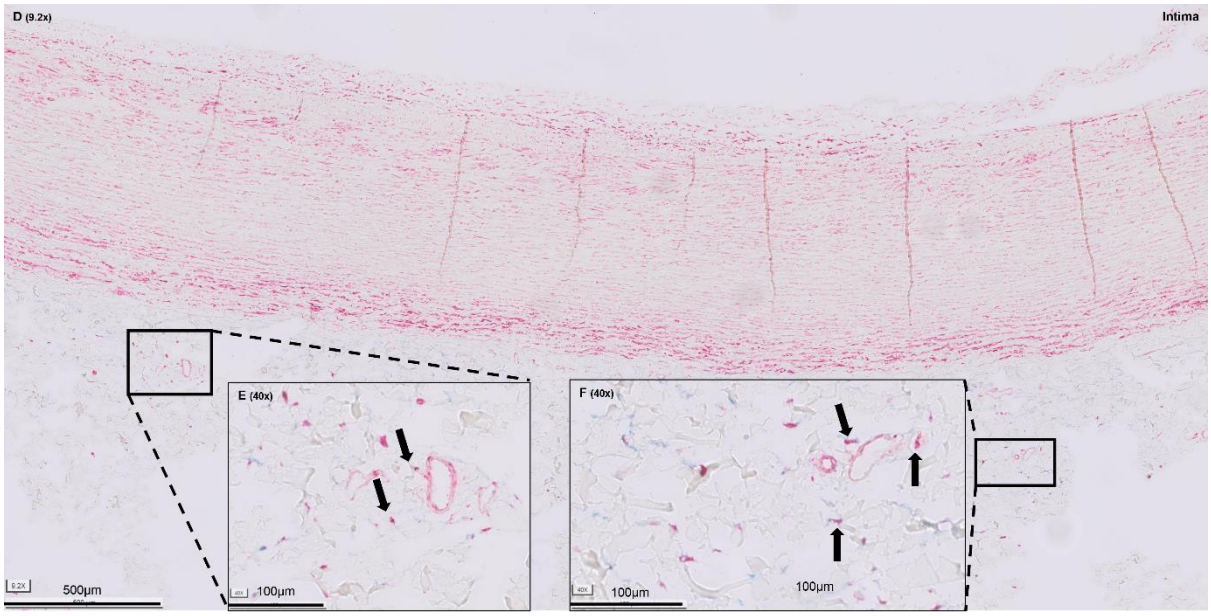


Figure S8.2: D. Double IHC staining of CD34 (in *blue*) and Vimentin (in *red*) on LFA. The inserts (**E/F**) show solitary double Vimentin^{+}/CD34^{+} cells in the vicinity of the vasa vasora (in purple; **arrows**).

Figure S9. Fibrocytes in luminal vascular repair sites.

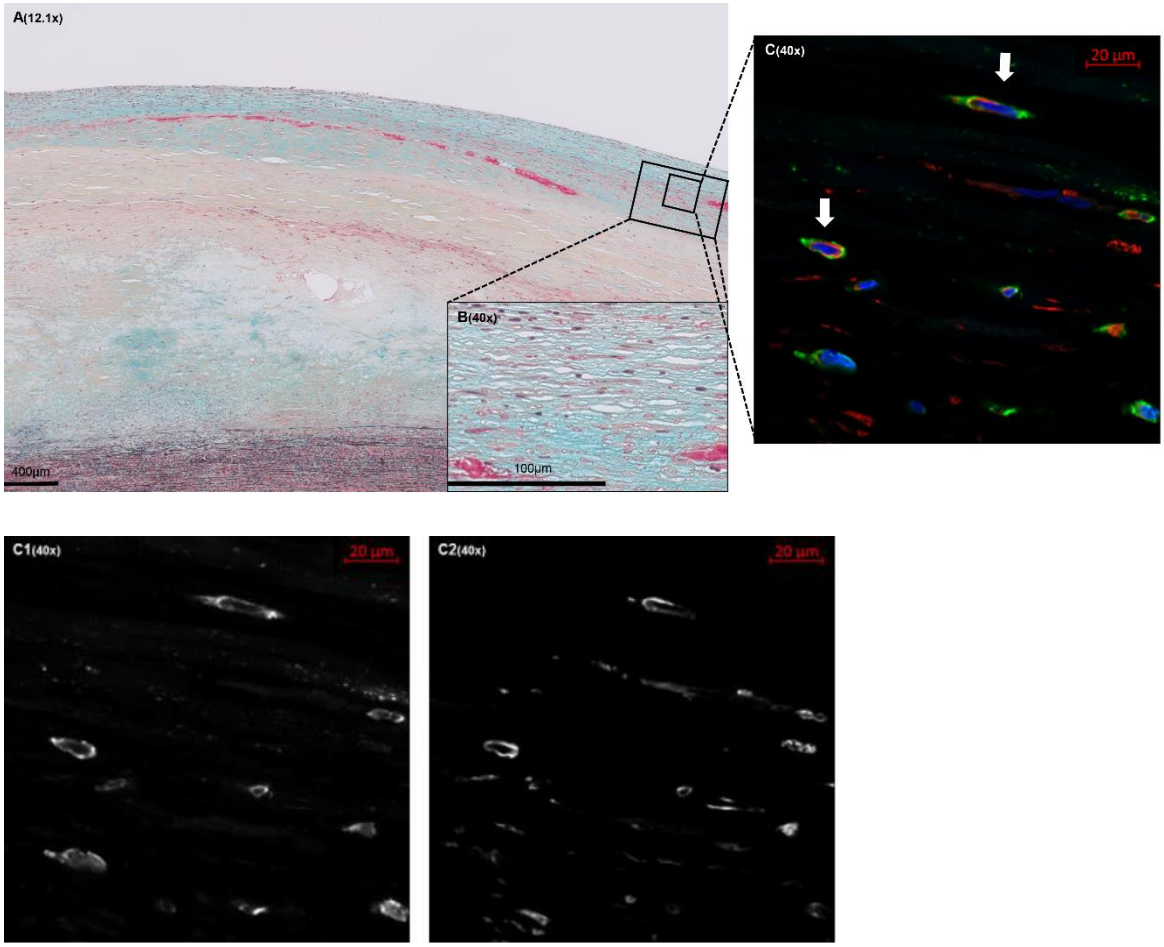


Figure S9.1: **A**. Healed Rupture (HR) stained by Movat pentachrome. Plaque consolidation (wound healing process) is characterized by a spindle-shaped mesenchymal-rich (**B;** nuclei in red) and proteoglycan-rich matrix (**B**; green-blue) cap covering the fibrotic remnants of the former cap (**A**; yellow region). **C**. Confocal images of spindle-shaped double Vimentin^{+} (in *green*; AF488)/ CD45^{+} (in *red*; AF647) in the proteoglycan-rich granulation tissue. **C1**. Shows single channel information for Vimentin and **C2** shows single channel information for CD45.

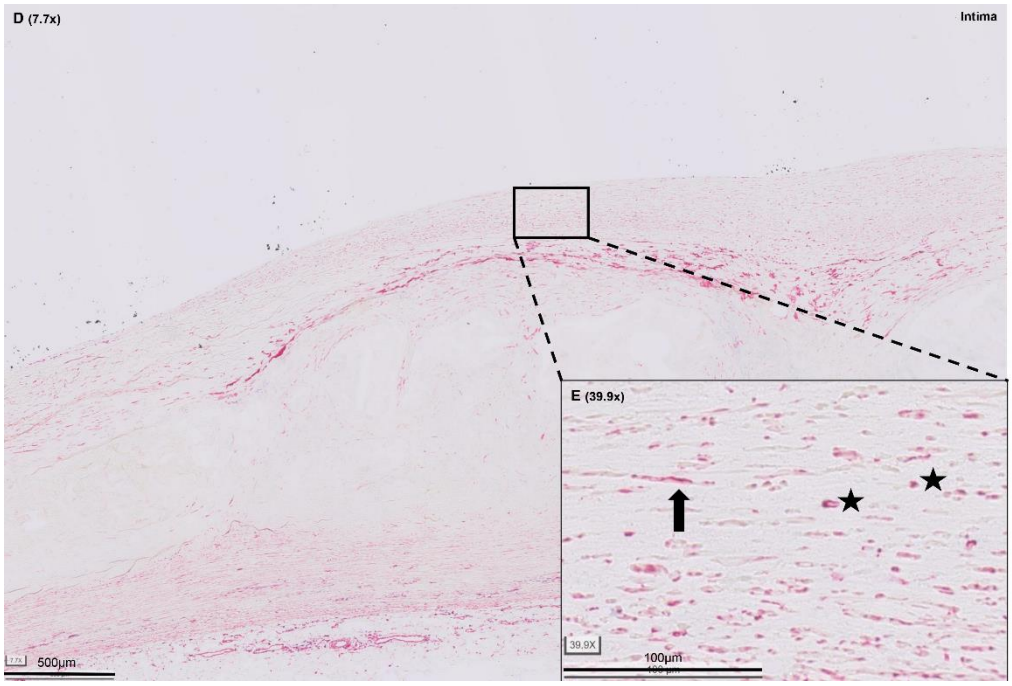


Figure S9.2: **D**. Late Fibroatheroma (LFA) double IHC stained for Vimentin (in *red*) and CD45 (in *blue*). **E**. In the cap of LFA, both spindle shaped double Vimentin^{+}/CD45^{+} (arrow) cells and round-shaped double Vimentin^{+}/CD45^{+} (stars) are present.

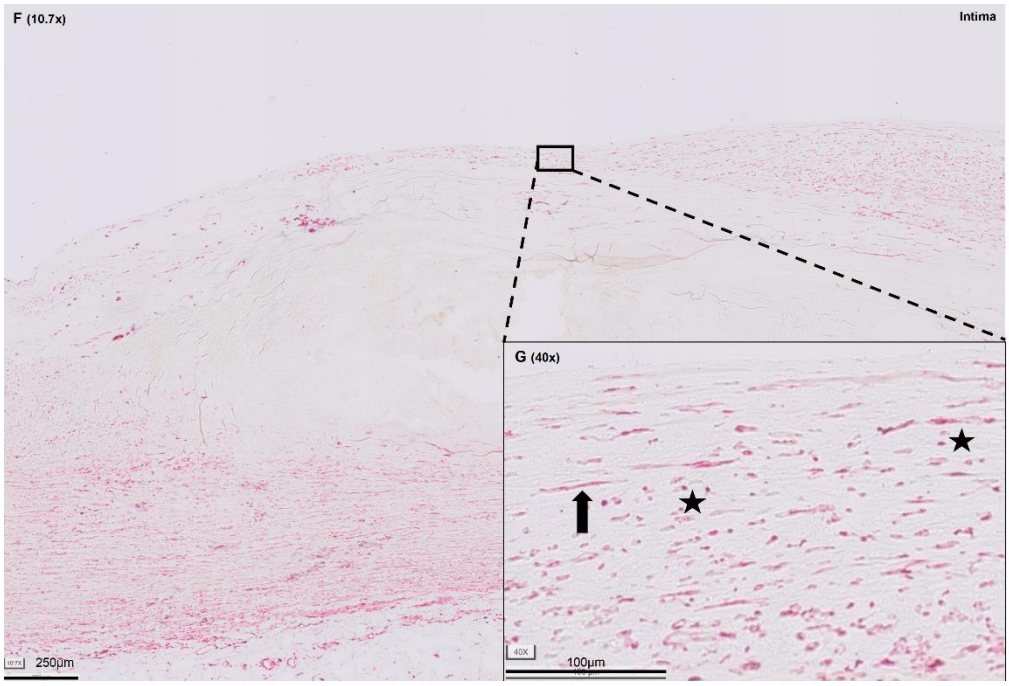


Figure S9.3: **F**. Fibrocalcific Plaque (FCP) double IHC stained for Vimentin (in *red*) and CD45 (in *blue*). **E**. In the neo-intima of FCP, both spindle shaped double Vimentin^{+}/CD45^{+} (arrow) cells and round-shaped double Vimentin^{+}/CD45^{+} (stars) are present.

Figure S10. Subset of elongated Smemb+ are synthetic.

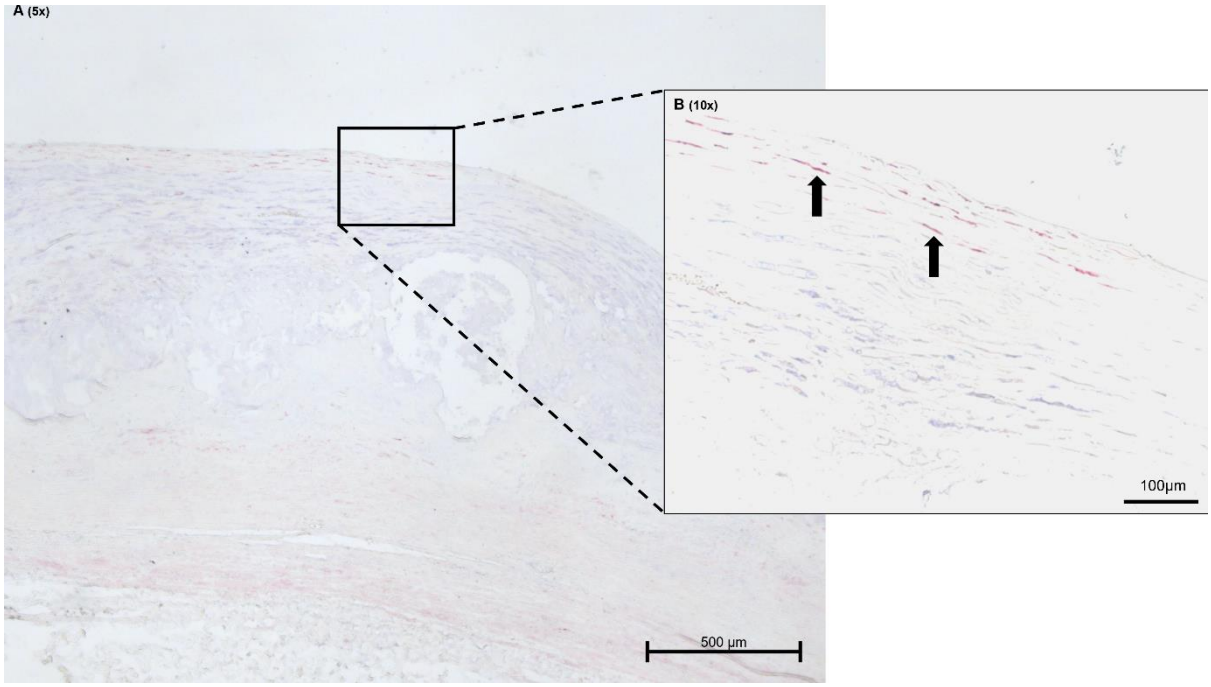


Figure S10: A. Late Fibroatheroma (LFA) section double IHC stained for Smemb (in *red*) and P4HB (in *blue*). Most Smemb+ elongated cells in the cap and shoulder region were found to also express the synthetic marker P4HB, but single Smemb+ elongated cells were also present in these regions (**B**; arrows).

Figure S11. Spatial distribution of h1-Calponin in progressive atherosclerosis.

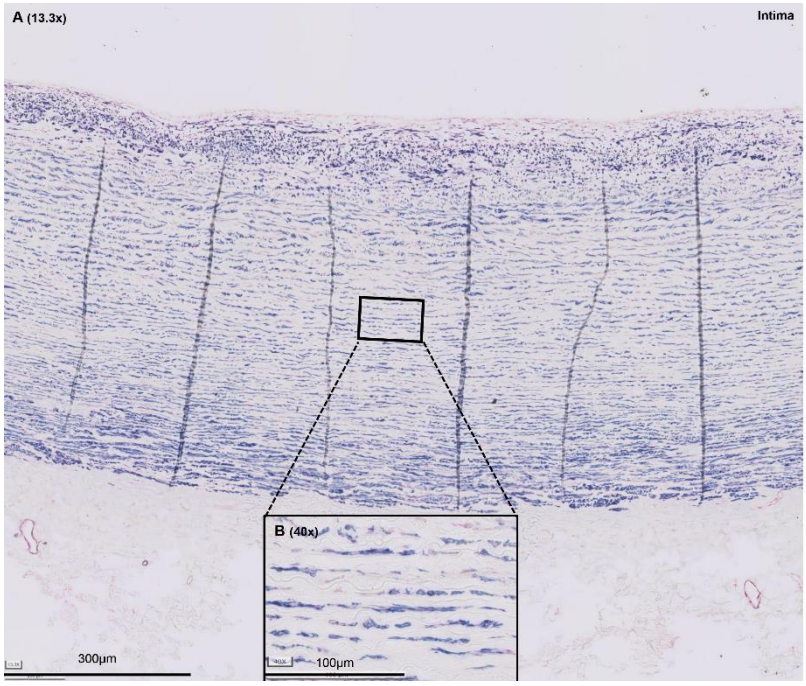


Figure S11.1: A. Adaptive Intimal Thickening (AIT) section double IHC stained for αSMA (in *red*) and h1-Calponin (in *blue*). In AIT, they show complete colocalization (**B**; in purple), except from the vasa vasora which are often single αSMA^{+} (**A**; in *red*).

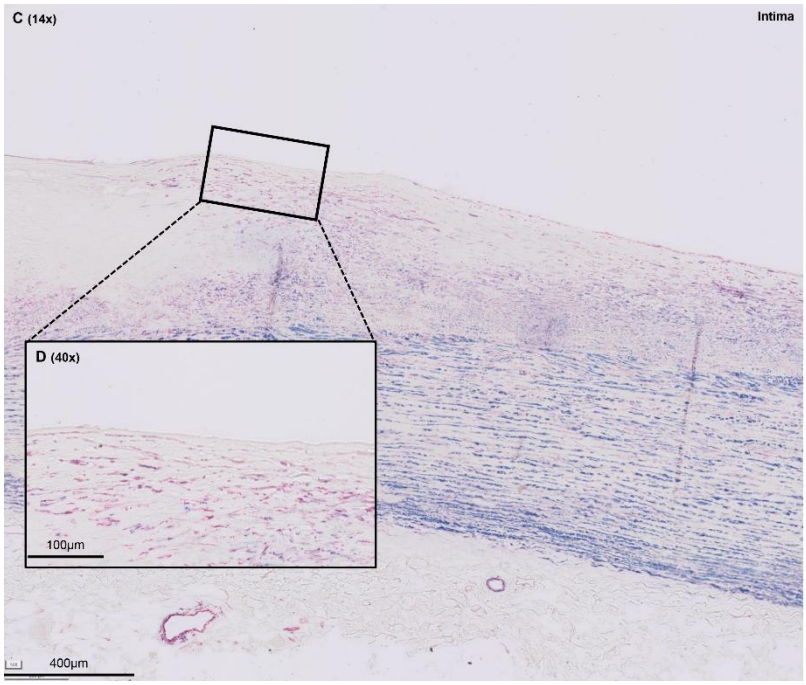


Figure S11.2: A. Late Fibroatheroma (LFA) section double IHC stained for αSMA (in *red*) and h1-Calponin (in *blue*). In LFA, there is a dissociation of the staining pattern for αSMA and h1-Calponin in the cap/shoulder region, reflected by single h1-Calponin^{-}/ αSMA^{+} cells (in *red*).

Figure S12. Tropomyosin, Telokin and Paxillin are not contractile cell specific.

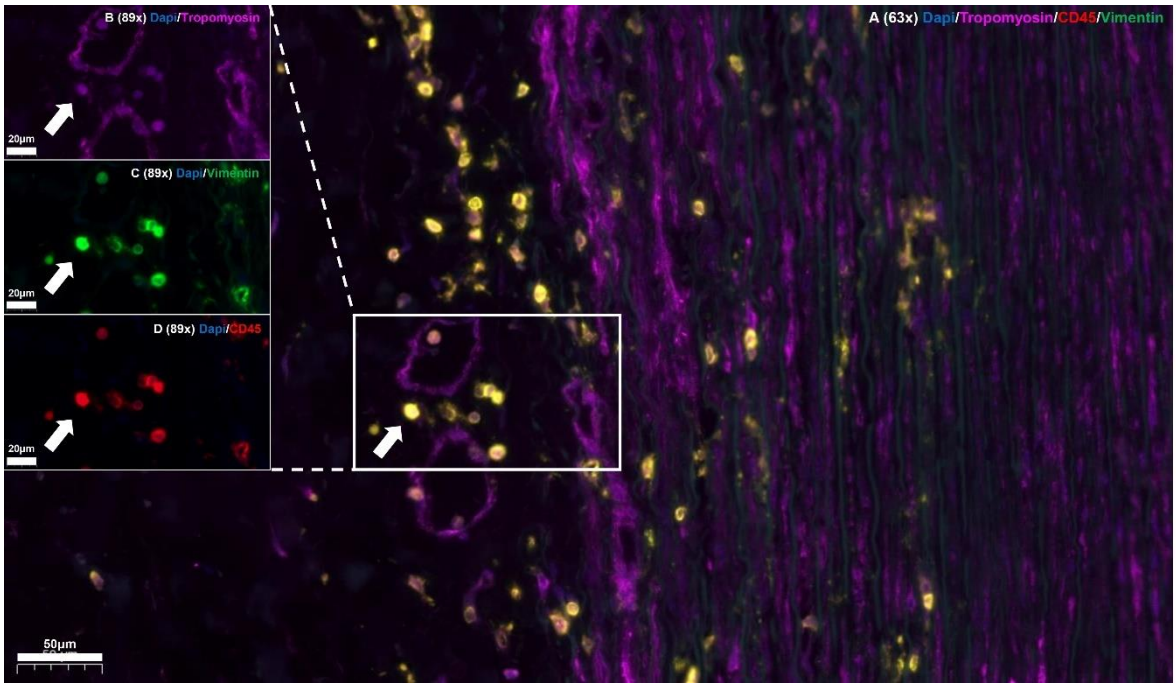


Figure S12.1: A. Triple staining of Tropomyosin (in *magenta*; AF647; Cy5-channel), Vimentin (in *green*; AF488; FITC-channel) and CD45 (in *red*; Vulcan Red; TRITC-channel) in the adventitia. The three left inserts show the single channel information for Tropomyosin (**B**), Vimentin (**C**) and CD45 (**D**), counterstained by Dapi (in *blue*). The vast majority of Tropomyosin+ cells were triple positive for Tropomyosin^{+}/Vimentin^{+}/CD45^{+}.

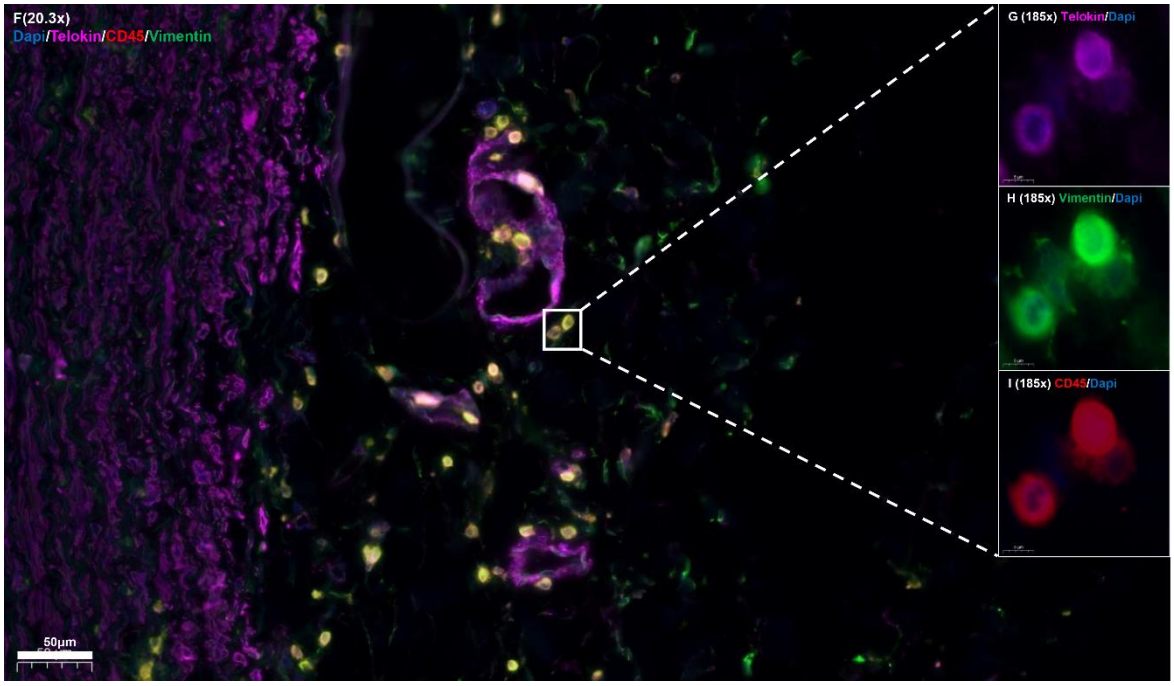


Figure S12.2: F. Triple staining of Telokin (in *magenta*; AF647; Cy5-channel), Vimentin (in *green*; AF488; FITC-channel) and CD45 (in *red*; Vulcan Red; TRITC-channel) in the adventitia. The three left inserts show the single channel information for Telokin (**G**), Vimentin (**H**) and CD45 (**I**), counterstained by Dapi (in *blue*). The vast majority of Telokin+ cells were triple positive for Telokin^{+}/Vimentin^{+}/CD45^{+}.

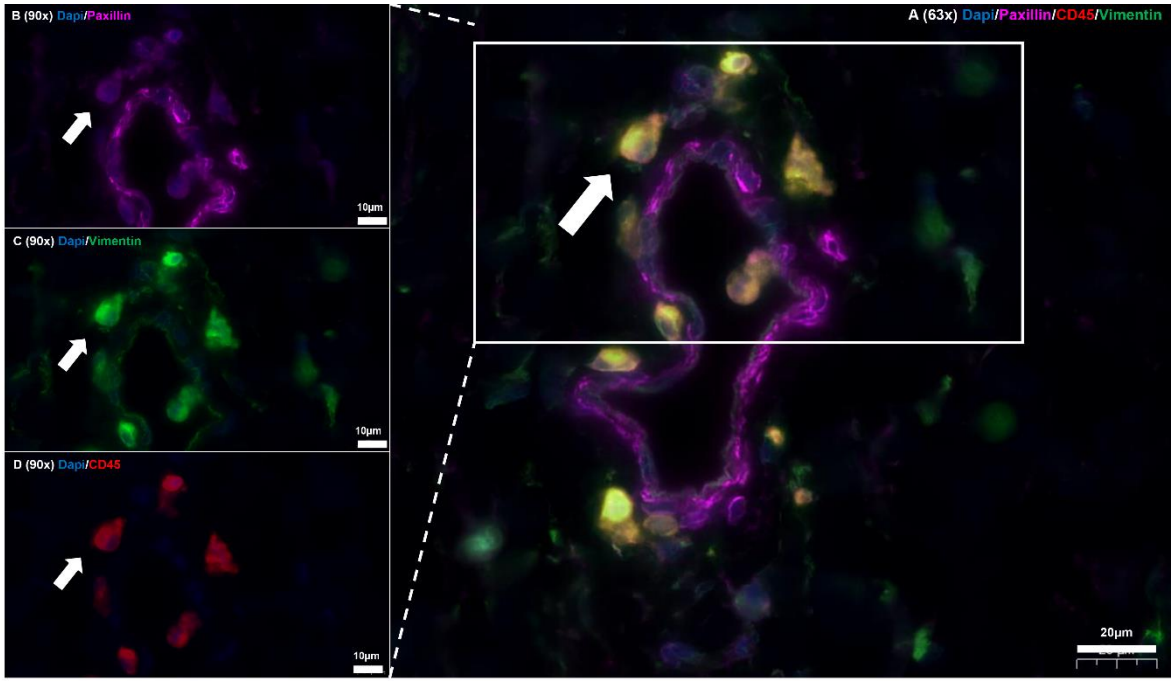


Fig S12.3: A. Triple staining of Paxillin (in *magenta*; AF647; Cy5-channel), Vimentin (in *green*; AF488; FITC-channel) and CD45 (in *red*; Vulcan Red; TRITC-channel) in the adventitia. The three left inserts show the single channel information for Paxillin (**B**), Vimentin (**C**) and CD45 (**D**), counterstained by Dapi (in *blue*). Although the vast majority of Paxillin$^+$ cells in the adventitia were triple positive for Paxillin$^+$/Vimentin$^+$/CD45$^+$, single Vimentin$^+$ cells were also present (**A**).

Figure S13. αSMA challenged as all-inclusive contractile marker.

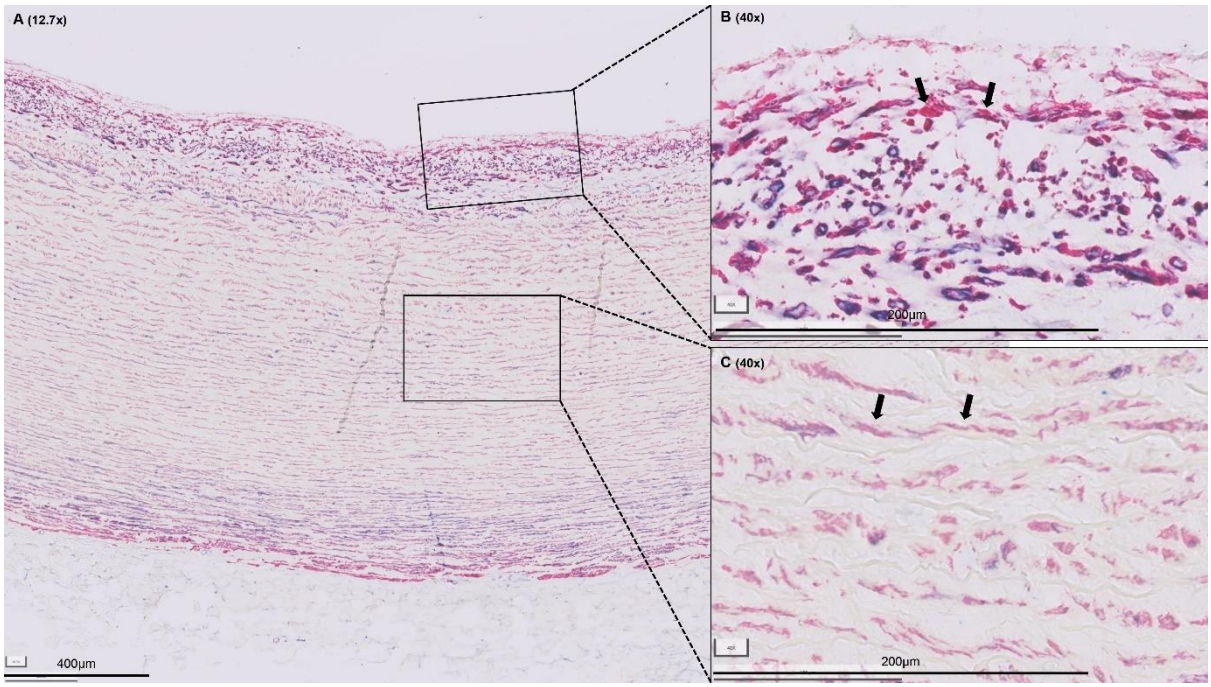


Figure S13.1: **A.** Adaptive Intimal Thickening (AIT) section double IHC stained for αSMA (in *red*) and Tropomyosin (in *blue*). In AIT, single αSMA^{+} cells (arrows in **B**) are present in the intima, as well as in the outer media. In the inner and medial media, all elongated cells are double αSMA^{+}/Tropomyosin^{+} *(in purple)*, but Tropomyosin is expressed in varying degrees (**C**. arrows: αSMA^{high}/Tropomyosin^{low}).

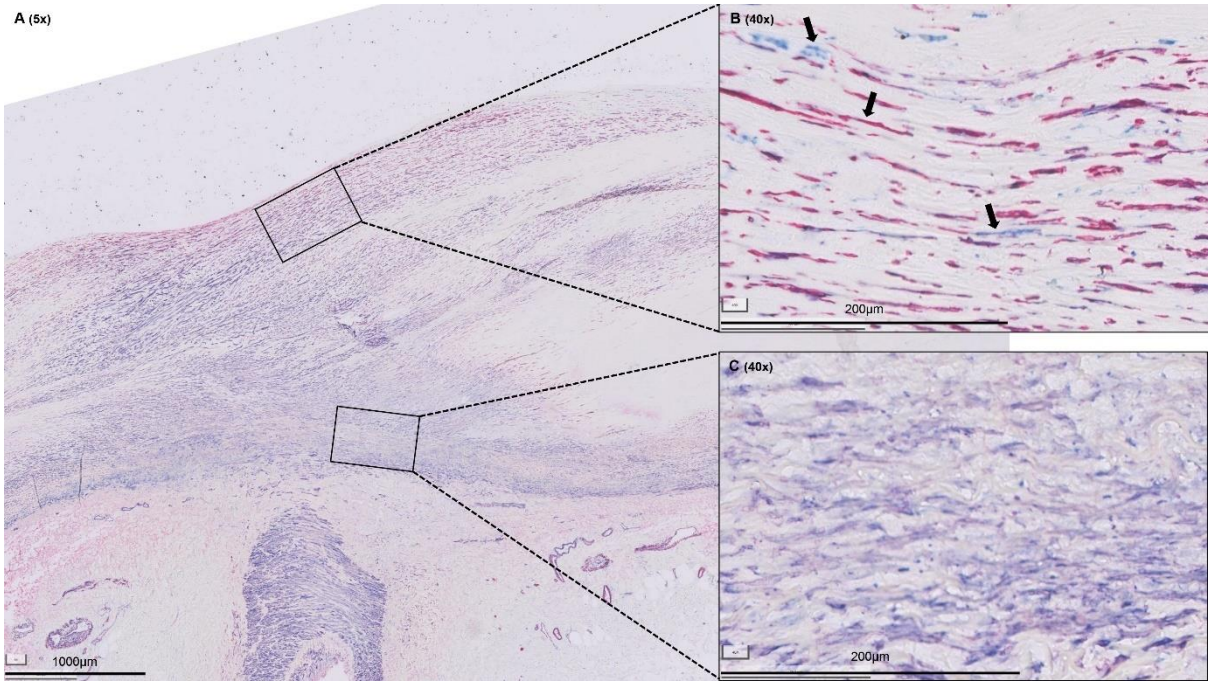


Figure S13.2: A. Late Fibroatheroma (LFA) section double IHC stained for αSMA (*red*) and Tropomyosin (*blue*). While in the media Tropomyosin and αSMA show complete overlap (**C**), in the cap and shoulder regions in LFA both single αSMA$^+$ cells and single Tropomyosin$^+$ cells are present (**B; arrows**).

Figure S14. Excluded synthetic and pro-inflammatory IHC markers.

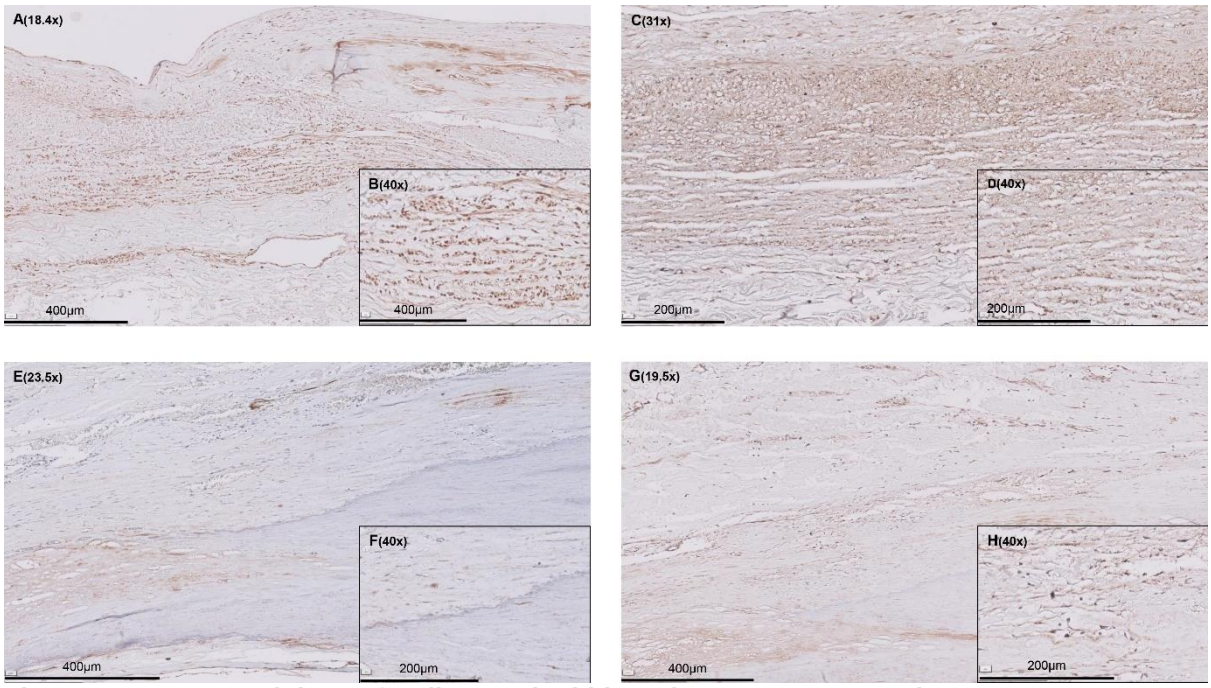


Figure S14.1: IHC Stainings of Collagen-I (Rabbit IgG), MBS502155, MyBioSource. In FCP tissue, significant non-specific staining was present, regardless of protein block usage and use of either a heat retrieval (**A**. Tris-EDTA, dilution 1:400) or an enzyme retrieval (**C**. Pepsin, dilution 1:400), similarly for AAA tissue (**E**. Tris-EDTA, dilution 1:400; **G**. Pepsin, dilution 1:400).

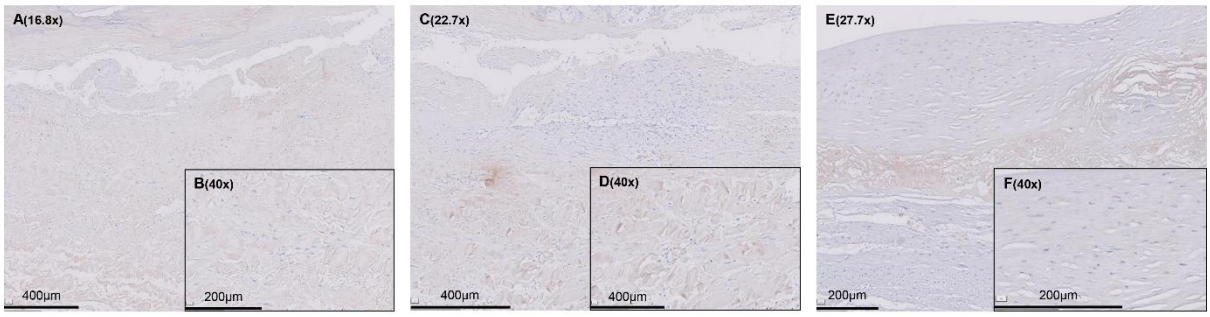


Figure S14.2: IHC Stainings of Collagen-I (Goat IgG), C7510-17K, USBIO. In AAA tissue, and even more outspoken in FCP tissue (**E**. Citrate, dilution 1:250), significant non-specific staining was present, regardless of protein block usage and use of either a heat retrieval (**A**. Citrate, dilution 1:250) or an enzyme retrieval (**C**. Pepsin, dilution 1:250).

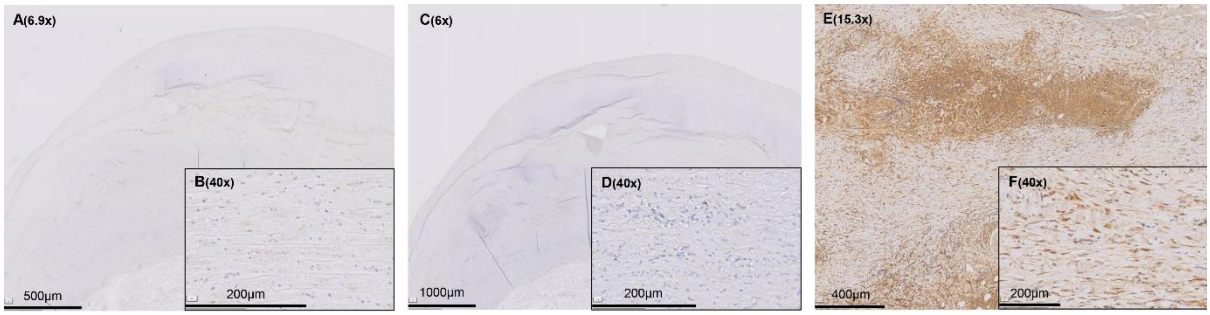


Figure S14.3: IHC Stainings of Pro-collagen-I (Rat IgG1), clone MAB1912, Millipore. In EFA tissue, regardless of antigen heat retrieval pH (**A**. Tris-EDTA, dilution 1:500; **B**. Citrate, dilution 1:500), there was little to no signal. However, in positive controls (**C**. AAA tissue, Tris-EDTA, dilution 1:500), a lot of non-specific staining was present, especially in lymphocyte infiltrates.

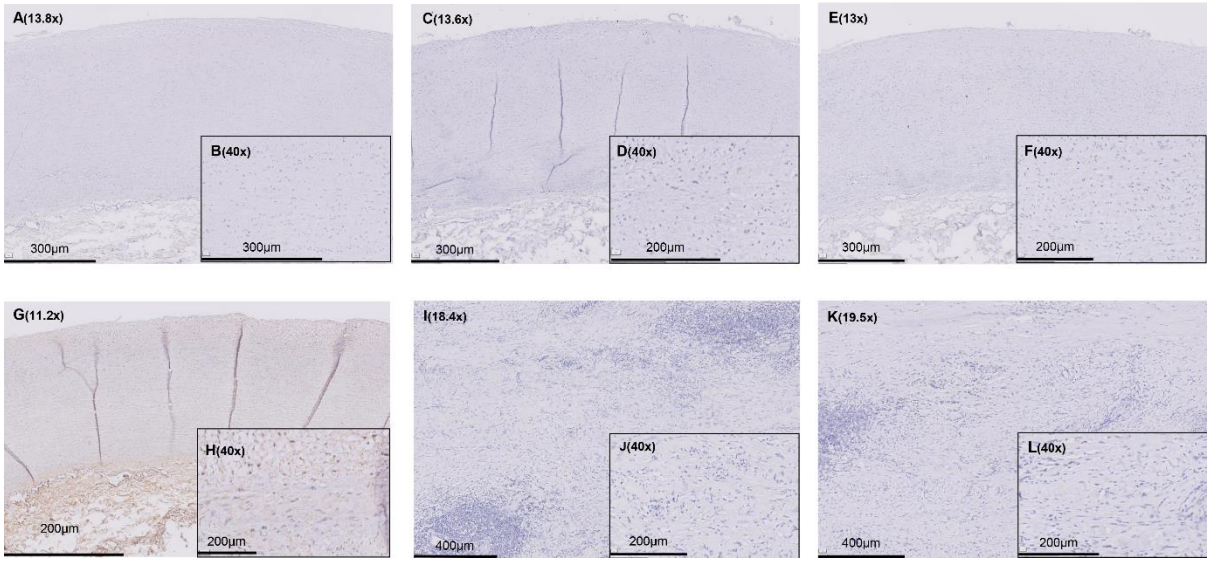


Figure S14.4: IHC Stainings of Pro-collagen-I (Mouse IgG1), clone PC8-7, Abnova. In EFA tissue, regardless of antigen heat retrieval pH (**A**. no retrieval, dilution 1:300; **C**. Tris-EDTA, dilution 1:300; **E**. Citrate, dilution 1:300), no protein expression was detected, confirmed by absence of staining in positive controls (**H**. AAA, no retrieval, dilution 1:300; **I**. AAA, Tris-EDTA, dilution 1:300). In contrast, in higher concentrations (**G**. dilution 1:100, EFA, no retrieval) there was significant background staining.

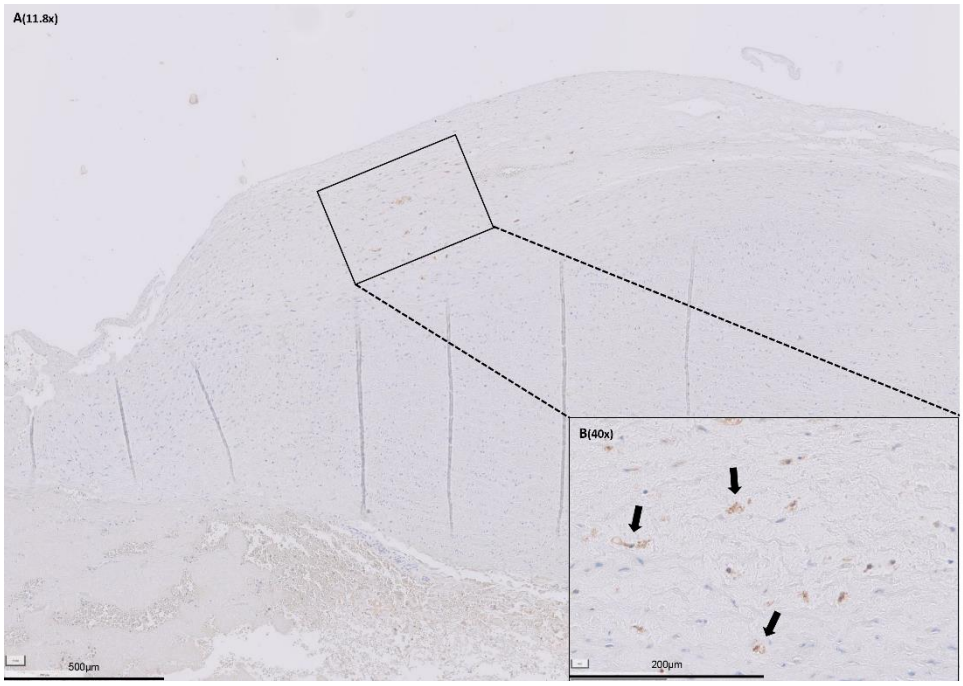


Figure S14.5: IHC Staining of Osteopontin (Goat IgG), AF1433, R&D Systems. A. (PIT (Pathological Intimal Thickening) tissue, Tris-EDTA, dilution 1:400). **B.** As Osteopontin is an ECM-protein (arrows), it is less convenient for cell phenotyping.

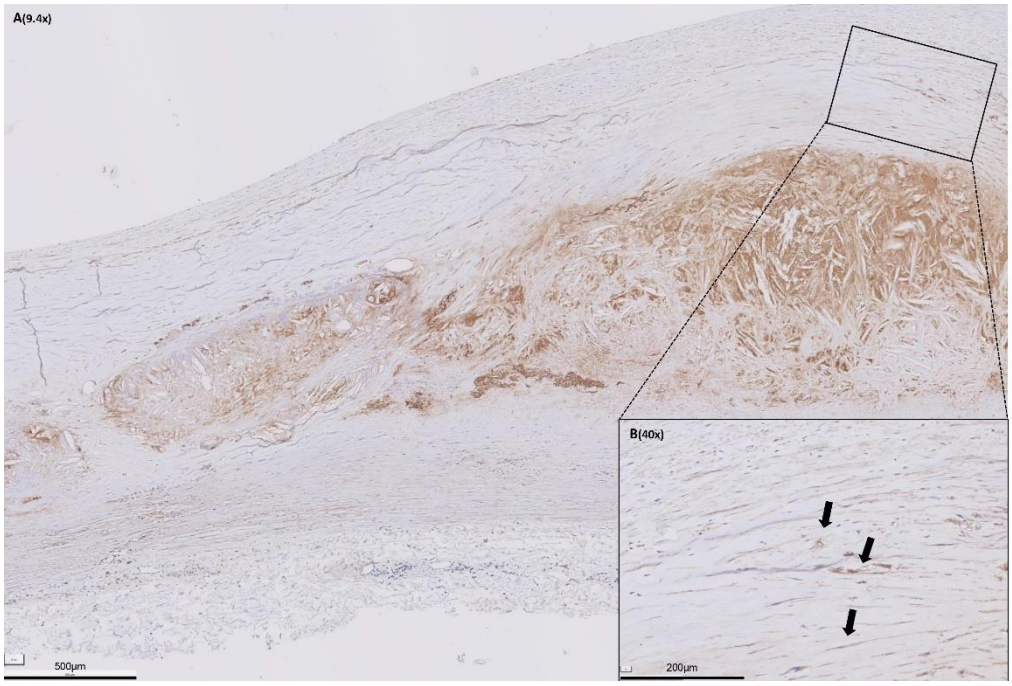


Figure S14.6: IHC Staining of Fibronectin (Mouse IgG1), clone FBN11, Thermofisher. A (LFA tissue, no retrieval, dilution 1:900)**. B.** As Fibronectin is an ECM-protein (arrows), it is less convenient for cell phenotyping.

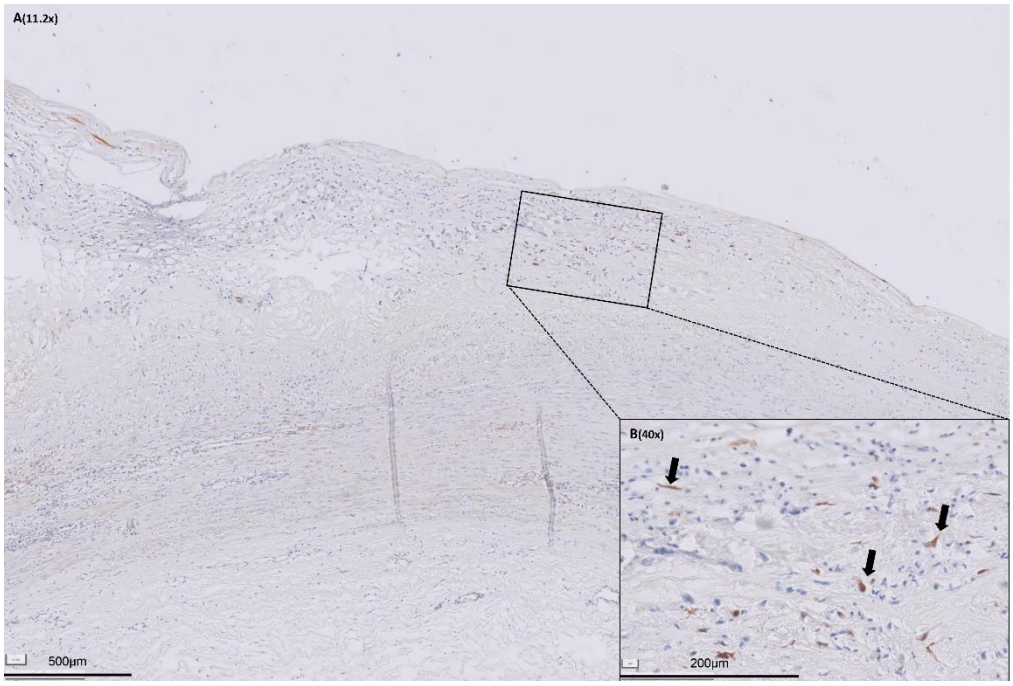


Figure S14.7: IHC staining of Laminin (Rabbit IgG), ab11575, Abcam. A. (Thin-cap Fibroatheroma tissue (advanced atherosclerosis), no retrieval, dilution 1:200)**. B.** As Laminin is an ECM-protein (arrows), it is less convenient for cell phenotyping.

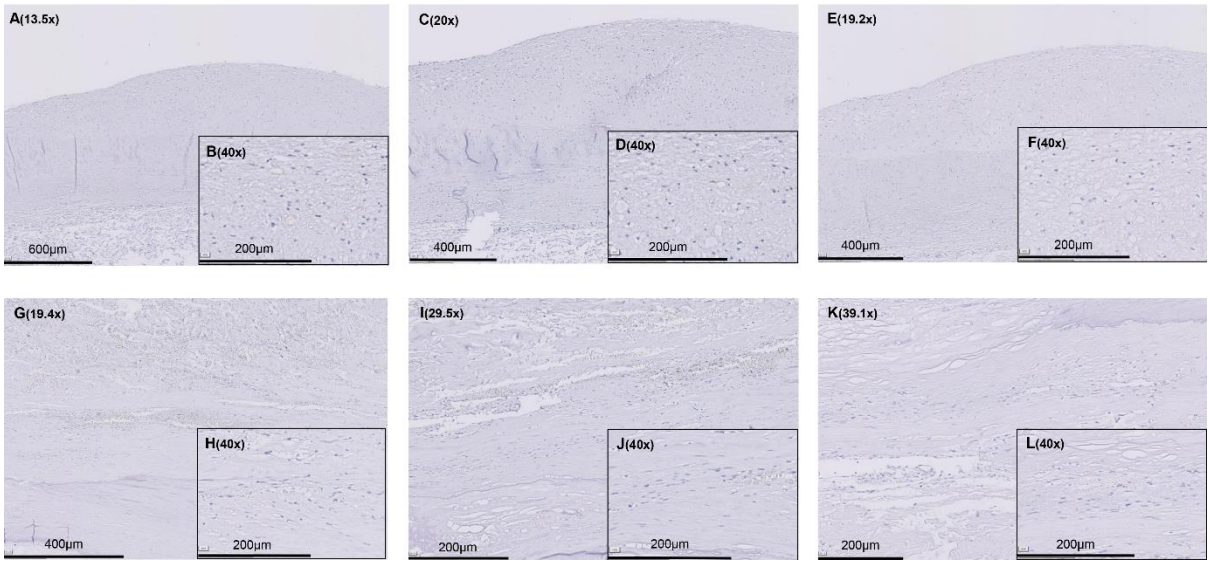


Figure S14.8: IHC staining of CRBP-1 (Rabbit IgG), ab11575, Abcam. In EFA (Early Fibroatheroma; advanced atherosclerosis) and AAA, no CRBP-1 signal was present, regardless of high primary antibody concentration and various antigen retrieval (**A**.EFA, Tris-EDTA, 1:30; **C**. EFA, Citrate, 1:30; **E**. EFA, Pepsin, 1:30; **G**. AAA, Tris-EDTA, 1:30; **I**. AAA, Citrate, 1:30; **K**. AAA, Pepsin, 1:30).

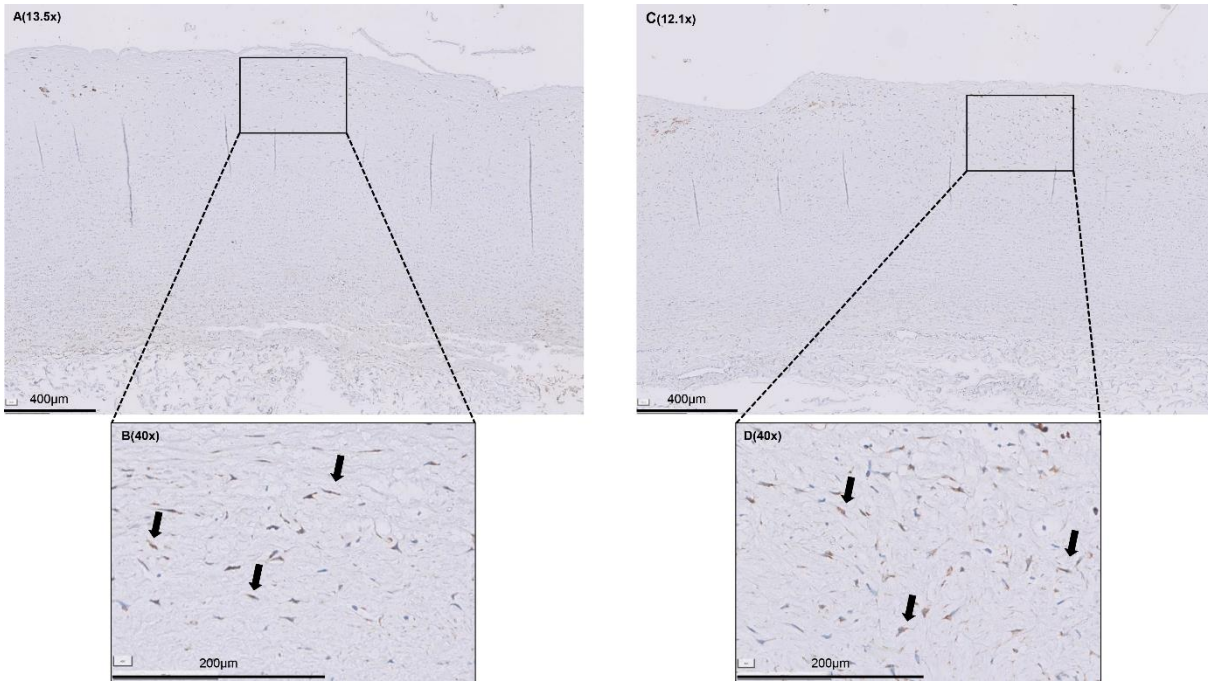


Figure S14.9: IHC stainings (n=1) of PDGFR-α (Rabbit IgG), ab61219, Abcam. Although PDGFR-α is expressed on the cell membrane, nuclear staining was observed (arrows in **B** (Citrate, dilution 1:400) and **D** (Tris-EDTA, dilution 1:400)). A small number of studies[326,327] has reported nuclear localization of the PDGFR-α, but those observations are based on IHC/IF, which makes it questionable whether it is really localized in the nucleus or whether it is background staining.

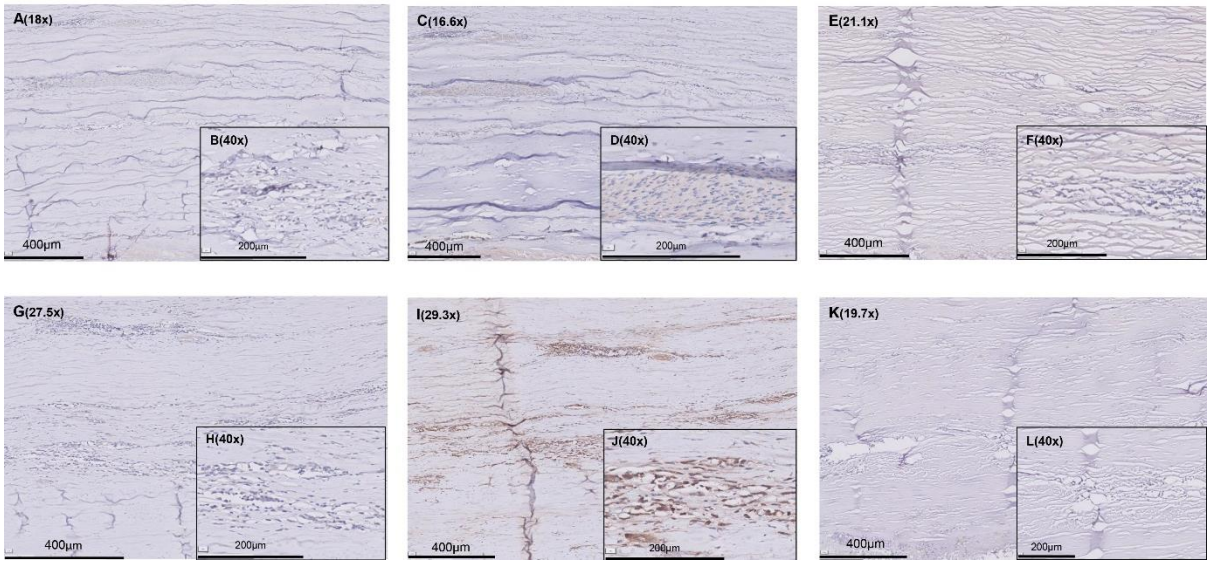


Figure S14.10: IHC Stainings of Phospho-NFκB p65 (Mouse IgG1), clone MCFA30, ThermoFisher. Although AAA is typically highly infiltrated by immune cells, there was weak staining of NFκB, regardless several antigen retrievals (**A**. Citrate, dilution 1:100; **C**. Tris-EDTA, dilution 1:100; **E**. Pepsin, dilution 1:100). In higher concentrations, more background staining was observed (**I**. Tris-EDTA, dilution 1:50). As NFκB is expressed intracellularly, the contribution of 0,1% Triton X-100 in PBS was also tested, although most nuclei are dissected in the 4 µ-sections (**G**. Citrate, Triton X-100, dilution 1:100; **K**. Pepsin, Triton X-100, dilution 1:100). However, more background (cytoplasmatic) staining was present if Triton X-100 was applied.

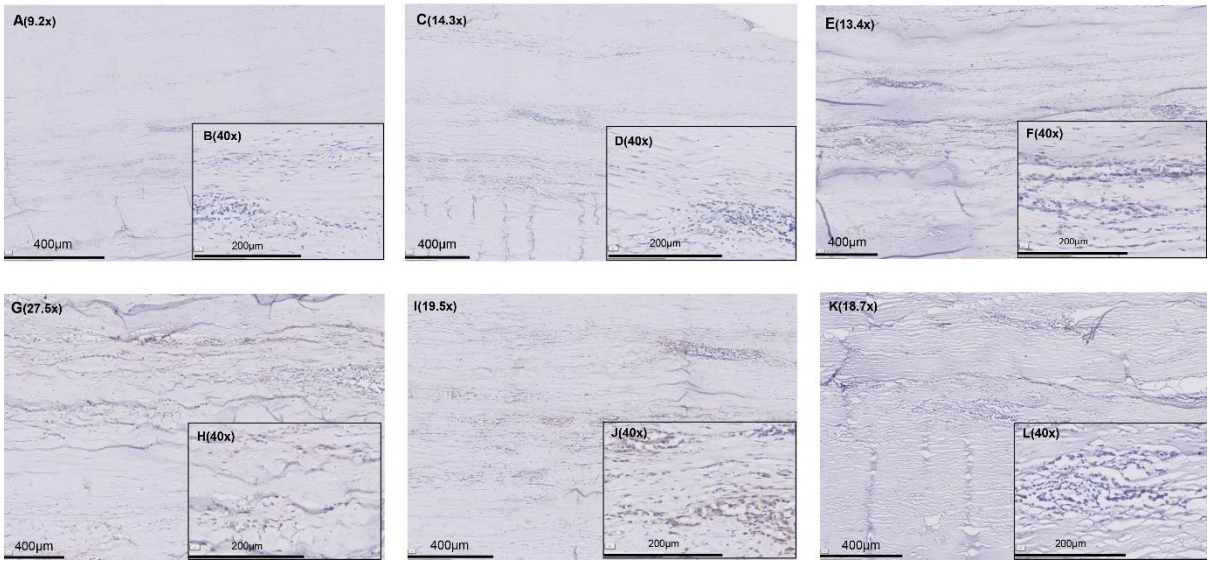


Figure S14.11: IHC Stainings of Phospho-NFκB 105 (Mouse IgG1), 178F3, Cell Signaling Technology.

Although AAA is typically highly infiltrated by immune cells, there was weak staining of NFκB, regardless several antigen retrievals (**A**. Tris-EDTA, dilution 1:100; **C**. Citrate, dilution 1:100; **E**. Pepsin, dilution 1:100). In higher concentrations, more background staining was observed (**K**. Tris-EDTA, dilution 1:50). As NFκB is expressed intracellularly, the contribution of 0,1% Triton X-100 in PBS was also tested, although most nuclei are dissected in the 4 µ-sections (**G**. Tris-EDTA, Triton X-100, dilution 1:100; **I**. Citrate, Triton X-100, dilution 1:100). However, more background (cytoplasmatic) staining was present if Triton X-100 was applied.

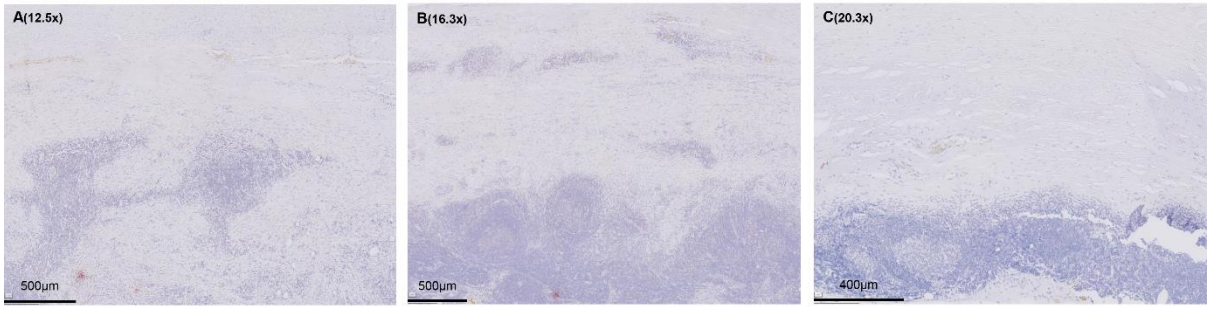


Figure S14.12: IHC Stainings of MCP-1 (Mouse IgG2b), 23002, R&D Systems.

Weak to no staining of MCP-1 in infiltrates of AAA tissue, regardless antigen retrieval (**A**. Tris-EDTA, dilution 1:100; **B**. Pepsin, dilution 1:100) and high primary antibody concentration (**C**. Tris-VEDTA, dilution 1:50).

Figure S15. Illustration of inflammatory cells in atherosclerosis.

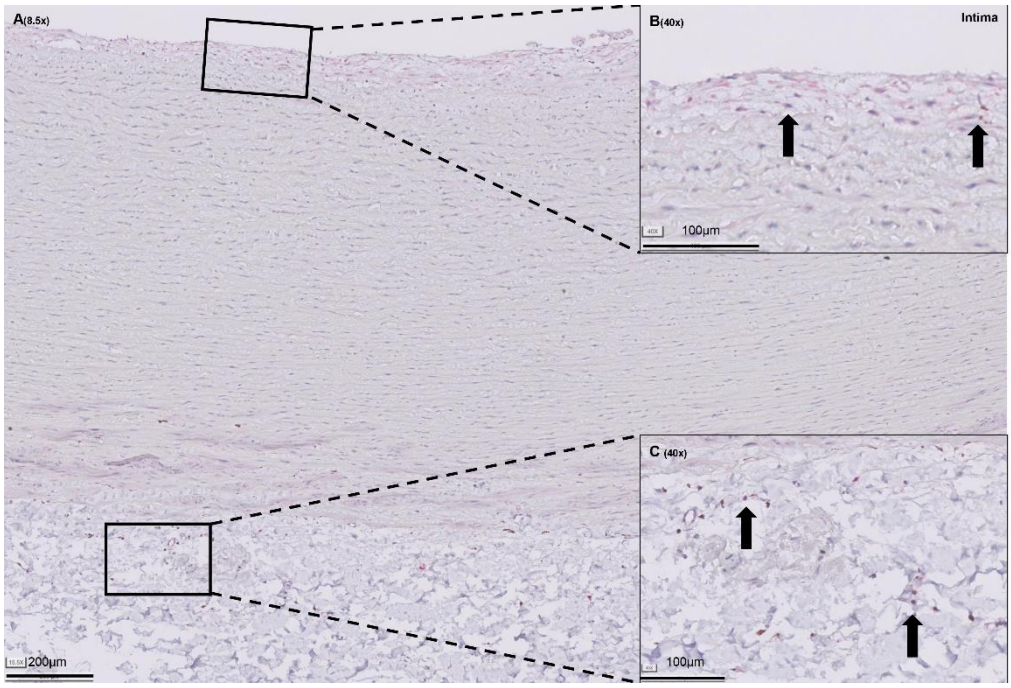


Figure S15.1: A. Double IHC staining for T-cells (CD4/CD8 co-staining; in *red*) and macrophages (CD68; in *brown*) in AIT reference sample (early atherosclerosis). **B.** Close up of T-cells (arrows) in the intima and **C**. close up of macrophages (arrows) in the adventitia.

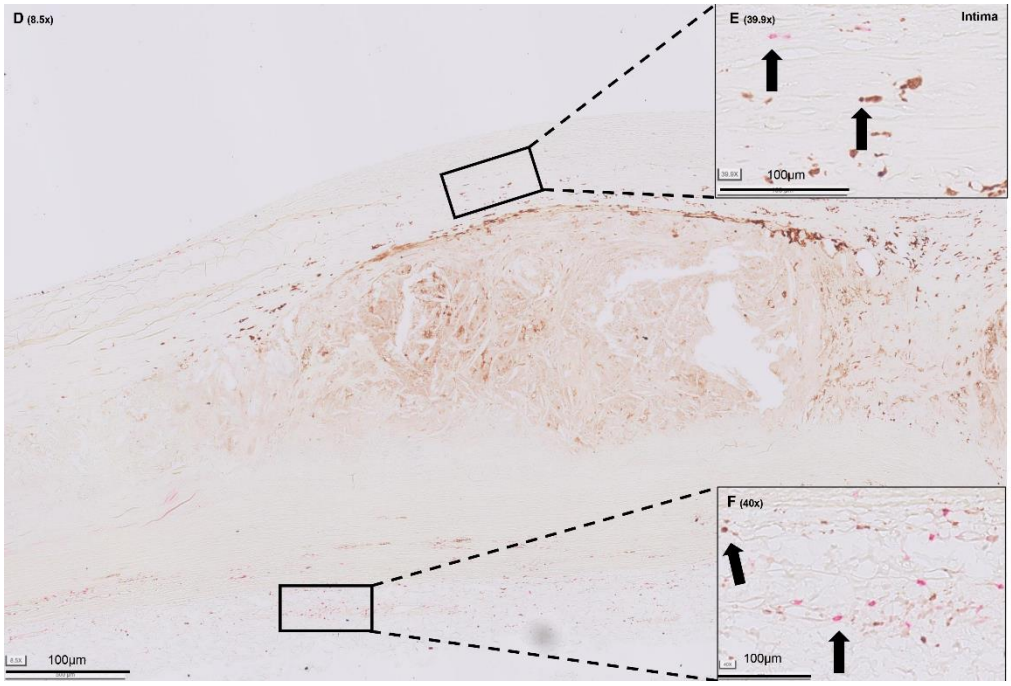


Figure S15.2: D. Double IHC staining for T-cells (CD4/CD8 co-staining; in *red*) and macrophages (CD68; in *brown*) in LFA reference sample (progressive atherosclerosis). **E.** Close up of T-cells (left arrow) and macrophages (right arrow) in cap area. **F**. Close up of macrophages (left arrow) and T-cells (right arrow) in adventitia.

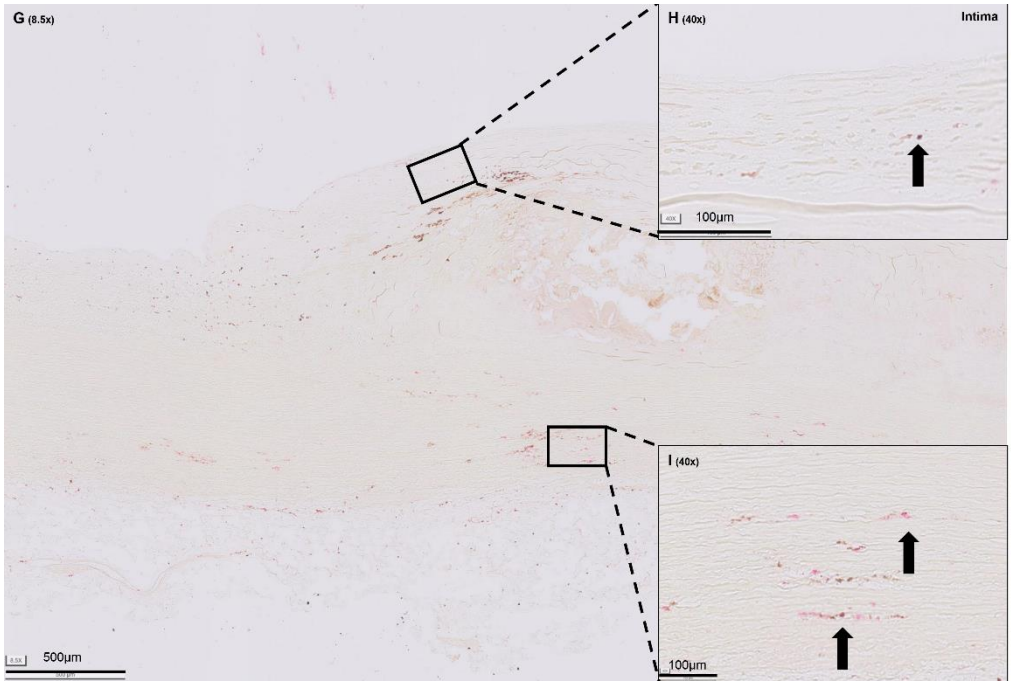


Figure S15.3: **G.** Double IHC staining for T-cells (CD4/CD8 co-staining; in *red*) and macrophages (CD68; in *brown*) in FCP reference sample (end-stage atherosclerosis). **H.** Close up of macrophages in the neo-intima. **I**. Close up of macrophages (left arrow) and T-cells (right arrow) in the adventitia.