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# Talin and Vinculin are Downregulated in Atherosclerotic Plaque; Tampere Vascular Study

Magdaléna von Essen[1*], Rolle Rahikainen[1*], Niku Oksala[2,3], Emma Raitoharju[2], Ilkka Seppälä[2], Ari Mennander[4], Thanos Sioris[4], Ivana Kholova[5], Norman Klopp[6], Thomas Illig[6,7,8], Pekka Karhunen[9], Mika Kähönen[10], Terho Lehtimäki[2 †], Vesa P. Hytönen[1 †]

[1] BioMediTech, University of Tampere and Fimlab Laboratories, Tampere, Finland
[2] Dep. of Clinical Chemistry, Fimlab Laboratories, Tampere University Hospital and School of Medicine, University of Tampere, Tampere, Finland
[3] Division of Vascular Surgery, Department of Surgery, Tampere University Hospital, Tampere, Finland
[4] Heart Center, Tampere University Hospital, Tampere, Finland
[5] Department of Pathology, Fimlab Laboratories, Tampere University Hospital and School of Medicine, University of Tampere, Tampere, Finland
[6] Hannover Unified Biobank, Hannover Medical School, Hannover, Germany.
[7] Institute of Human Genetics, Hannover Medical School, Hannover, Germany.
[8] Research Unit of Molecular Epidemiology, Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg, Germany.
[9] School of Medicine, University of Tampere and Fimlab Laboratories, Tampere University Hospital, Tampere, Finland
[10] Department of Clinical Physiology, Tampere University Hospital and School of Medicine, University of Tampere, Tampere, Finland.

* , † These authors contributed equally to this work

Short Title: Talin and Vinculin in human atherosclerosis

Corresponding author:
Vesa Hytönen
BioMediTech,
Lääkärinkatu 1, FI-33520 Tampere, Finland
email: vesa.hytonen@uta.fi
Phone: +358401901517

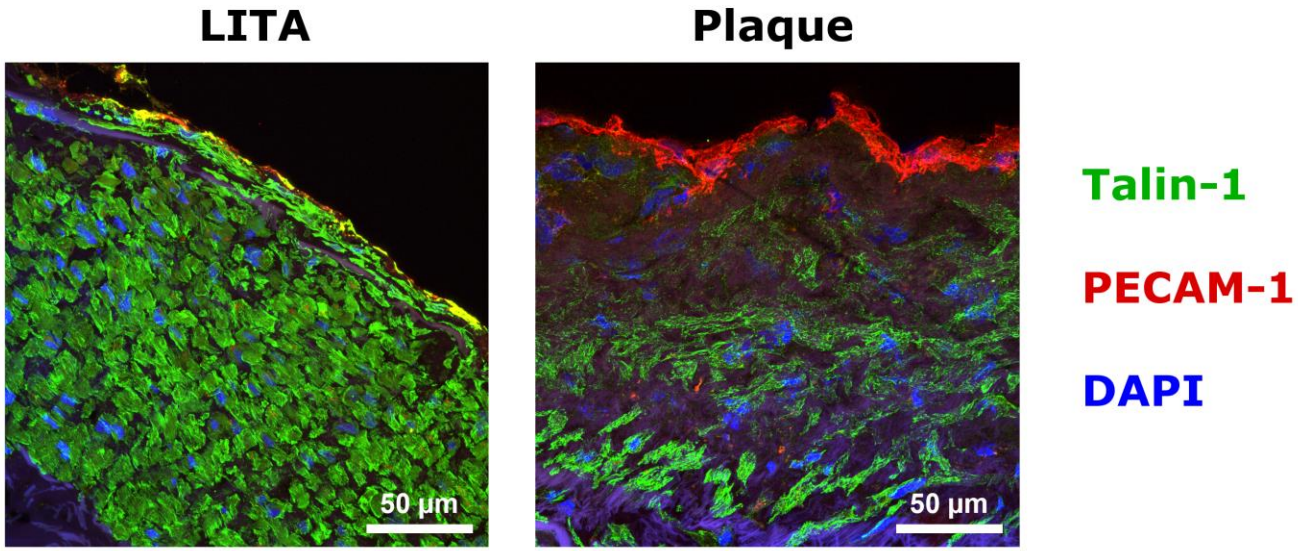
## *Abstract*

Background. Focal adhesions (FA) play an important role in the tissue remodeling and in the maintenance of tissue integrity and homeostasis. Talin and vinculin proteins are among the major constituents of FAs contributing to cellular well-being and intercellular communication.

Methods and Results. Microarray analysis (MA) and qRT-PCR low-density array were implemented to analyze talin-1, talin-2, meta-vinculin and vinculin gene expression in circulating blood and arterial plaque. All analyzed genes were significantly and consistently downregulated in plaques (carotid, abdominal aortic and femoral regions) compared to left internal thoracic artery (LITA) control. The use of LITA samples as controls for arterial plaque samples was validated using immunohistochemistry by comparing LITA samples with healthy arterial samples from a cadaver. Even though the differences in expression levels between stable and unstable plaques were not statistically significant, we observed further negative tendency in the expression in unstable atherosclerotic plaques. The confocal tissue imaging revealed gradient of talin-1 expression in plaque with reduction close to the vessel lumen. Similar gradient was observed for talin-2 expression in LITA controls but was not detected in plaques. This suggests that impaired tissue mechanostability affects the tissue remodeling and healing capabilities leading to development of unstable plaques.

Conclusion. The central role of talin and vinculin in cell adhesions suggests that the disintegration of the tissue in atherosclerosis could be partially driven by downregulation of these genes, leading to loosening of cell-ECM interactions and remodeling of the tissue.


## Graphical abstract



## Highlights

1. Talin and vinculin are downregulated in atherosclerotic plaques in all arterial beds
2. Expression of talin-1 in plaque is severely downregulated close to vessel lumen
3. Talin-2 expression in LITA shows a gradient towards vessel lumen which is absent in the plaque
4. The talin and vinculin expression may be further reduced in with progression of the disease, i.e. in later stages of plaque development and thrombosis
5. Talin and vinculin downregulation in atherosclerotic plaque causes tissue disorganization and may lead to reduced tolerance against mechanical impacts and impaired healing processes


## *Keywords*

Mechanobiology, atherosclerosis, focal adhesion, talin, vinculin


## *Abbreviations*

| | | |
|---|---|---|
| BMI | - | Body Mass Index |
| CAD | - | Coronary Artery Disease |
| ECM | - | Extracellular matter |
| FA | - | Focal Adhesion |
| FC | - | Focal Complex |
| fc | - | fold change |
| HUVEC | - | Human Umbilical Vein Endothelial Cell |
| ICAM | - | Intercellular Adhesion Molecule |
| LDA | - | Low Density Array |
| LITA | - | left internal thoracic artery |
| MA | - | Microarray Analysis |
| MΦ | - | Macrophage |
| PECAM | - | Platelet endothelial cell Adhesion Molecule |
| SMC | - | Smooth Muscle Cell |
| TVS | - | Tampere Vascular Study |
| VBS | - | Vinculin Binding Site |
| VCAM | - | Vascular Cell Adhesion Molecule |
| FFPE | - | Formalin-Fixed, Paraffin-Embedded |
| HE | - | Hematoxylin-Eosin |

# Introduction

Atherosclerosis is a disease of the vasculature with a complex etiology. Risk factors include age, sex, family history, dyslipidemia, high blood pressure and high body mass index (BMI), stress and dietary factors**.** The disease develops over a long time period and may remain asymptomatic over decades. It is characterized by chronic inflammation of the arterial wall, by infiltration of macrophages (MΦ) and accumulation of oxidized low-density lipoproteins leading to MΦ conversion to foam cells [1].

The vasculature is continuously exposed to cyclical fluctuations of blood flow, pressure and fluid shear stress and also exhibits diurnal variation. The blood mechanical impacts of varying magnitudes exert significant influences on physiological and pathophysiological processes [2] [3] [4]. For illustration, veins and arteries are composed of several tissue layers with different cell and extra-cellular matter (ECM) content. This cell and ECM composition determines the tissue characteristics in terms of physicochemical properties [5] [6]. Hence, each vessel layer possesses different ability to withstand, produce or transduce mechanical forces [5]. The mechanical pressure sensed by the endothelial cells is transferred from the extracellular space through the actin cytoskeletal network towards the nucleus [7] [8].

To date, a number of genes implicated in cellular mechanostability and their altered expression has been associated with the progress of atherosclerosis. For example, ADAM metalloprotease disintegrins have been linked with cell-cell/surface adhesion and inflammation progression in the atherosclerotic plaque [9]. Moreover, the expression levels of integrin and kindlin family proteins were found to be altered in progressing atherosclerotic plaques [10]. Intergrin and kindlin proteins support leukocyte adhesion, transendothelial migration, platelet aggregation and thrombosis. Furthermore, integrins and kindlins are together with talin and vinculin among the major components of focal adhesions (FA). FAs are key attachments between cells and ECM and play an important role in cell morphology, differentiation, locomotion and intercellular communication. FAs are crucial for the tissue remodeling, integrity and homeostasis through the maintenance of intercellular gaps and cell adhesion supervision.

Talin is a large flexible protein [11] binding to transmembrane integrins (N-terminal FERM domain) [12] and to cytoskeletal actin (C-terminal rod) [13] providing a vital link between the intra- and extracellular space and allowing the communication between the ECM and nucleus [8]. Talin plays a significant role in the actin filament assembly and in spreading and migration of various cell types. During the adhesion maturation, talin recruits vinculin to crosslink with F-actin filaments and stabilize the adhesion complex. For this purpose talin rod contains several binding sites for vinculin [14]. Vinculin binding sites (VBSs) are buried inside the structural bundles and require a major conformational change in the bundle organization prior to vinculin binding [15]. Mechanical force has been suspected to mediate such domain reorganization and talin-vinculin binding [16] [17]. Talin interacts with several ligands making it a vital component of numerous mechanosensor and chemical signaling pathways [18] [19] [20] [21].

Vinculin is a cytoskeletal protein crosslinking talin and F-actin. Vinculin is ubiquitously expressed with high expression in skeletal, cardiac and smooth muscle. Vinculin head at the N-terminal end binds to talin's VBSs [22]. Vinculin tail at the C-terminal end binds F-actin [23]. Also other important interactions of vinculin have been recognized, for example with paxilin [24] and α-actinin [25]. These ligands make vinculin an important contributor to focal adhesion complex, as well as to the cytoskeletal assembly and stability.

The progress and the causatives of atherosclerosis have been intensively investigated during the past decades. Still, the mechanisms behind the disease development are not fully understood. In more detail, the mechanical impact of shear stress on the cell and tissue integrity has risen to attention only recently. We hypothesize that the cellular mechanostability and maintenance of tissue integrity through focal adhesions is an important factor in all stages of atherosclerotic plaque development. We

speculate that the function of focal adhesions is compromised by altered expression of cell adhesion proteins talin and vinculin in atherosclerotic plaque as compared to non-atherosclerotic vessel wall.

In this work we followed talin and vinculin expression in atherosclerotic plaque samples collected in ongoing Tampere Vascular Study (TVS) series. Gene expression in carotid, abdominal aortic and femoral plaque samples was compared to expression values in left internal thoracic artery (LITA) controls. Expression levels were determined by microarray analysis and low-density qRT-PCR-array. Results are supported by smooth muscle cell (SMC) and macrophage (MΦ) marker co-expression analysis. The tissue localization of talin and vinculin was investigated by confocal immunofluorescence study.

# Methods

## Vascular Samples

Arterial sample series from Tampere Vascular Study (TVS) [9] [26] [10] including samples from femoral, carotid and abdominal aortic regions were obtained during open vascular procedures between 2005 and 2015. The patients fulfilled the following inclusion criteria: (1) carotid endarterectomy performed because of asymptomatic or symptomatic and hemodynamically significant carotid stenosis (>70%); (2) femoral or (3) aortic endarterectomy with aortoiliac or aortobifemoral bypass based on symptomatic peripheral arterial disease. The left internal thoracic artery (LITA) controls were obtained during coronary artery bypass surgery due to coronary artery disease (CAD). The samples were collected from patients subjected to open vascular surgery in the Division of Vascular Surgery and Heart Center, Tampere University Hospital. The patient's denial to participate in the study was used as a measure of exclusion. The vascular samples were classified according to American Heart Association recommendation [27]. The type V and VI atherosclerotic lesions were further histologically classified as stable and unstable according to the presence of fissure, rupture, hemorrhage or thrombosis. Gene expression was analyzed from carotid (n=29), abdominal aortic (n=15), and femoral (n=24) plaques (*cases*) and compared to atherosclerosis-free LITA samples (n=28) (*controls*). The study has been approved by the Ethics Committee of Tampere Hospital District. All studies were conducted according to the declaration of Helsinki, with the informed consent from individual patient involved.

## Whole Blood and Circulating Monocyte Fractions

TVS whole blood and monocyte fractions were collected during 2008. The angiographically verified samples were selected from a larger population-based cross-sectional study [28] collected between 2001 and 2004 comprising patients subjected to an exercise test at Tampere University Hospital (treatment according to the Finnish Current Care Guidelines). RNA was isolated from the whole blood and monocyte fractions of individuals with CAD (n=55) and without coronary artery lesions (n=45). Patient history data were based on hospital records and patient interviews. These data covered the demographics such as age, sex, weight, lifestyle information and classical cardiovascular risk factors and symptoms.

## RNA Isolation and Microarrays

The fresh arterial tissue samples were soaked in RNALater solution (Ambion Inc, Austin, TX) and isolated with Trizol reagent (Invitrogen, Carlsbad, CA) and the RNAEasy Kit with DNase Set (Qiagen, Valencia, CA). From the whole blood fraction, the RNA was isolated with PAXgene tubes (BD, Franklin Lakes, NJ) and PAXgene Blood RNA Kit (Qiagen) with DNase Set. Peripheral mononuclear cells were isolated from the whole blood samples by Ficoll-Paque density-gradient centrifugation (Amersham Pharmacia Biotech UK Limited, Buckinghamshire, England). Total RNA was then extracted using RNeasy Mini Kit (Qiagen). Manufacturers' instructions were followed in all isolation protocols. The quality of the RNA samples was evaluated spectrophotometrically and stored at –80°C.

The expression levels of arterial and whole blood samples were analyzed with Illumina HumanHT-12 v3 Expression BeadChip (Illumina, San Diego, CA) analyzing 47 000 transcripts of all known genes, gene candidates, and splice variants. The microarray experiments for the monocyte RNA were performed using Sentrix Human-6 Expression BeadChips analyzing >46 000 transcripts (Illumina). Both arrays were run according to given instructions by the manufacturer and scanned with the Illumina iScan system. Further details of the methodology can be found in work by Turpeinen et al. [29].

## Microarray Data Analysis

After background subtraction, raw intensity data were exported using the Illumina GenomeStudio software. Raw expression data were imported into R software, log2 transformed and normalized by the locally estimated scatterplot smoothing normalization method implemented in the R/Bioconductor package Lumi. Locally estimated scatterplot smoothing normalization returned the best accuracy to detect differentially expressed genes in comparison with quantitative reverse transcription polymerase chain reaction using the control artery (LITA) and atherosclerotic plaque samples from the TVS study [30]. All samples fulfilled following data quality control criteria; detection of outlier arrays based on the low number of robustly expressed genes and hierarchical clustering. Probes were considered robustly expressed if the detection was $p<0.05$ for minimum of 50% of the samples in the data set. *TLN1* (microarray element probe ILMN_1696643), *TLN2* (microarray element probe ILMN_1700042) and *VCL* (transcript variant 1, microarray element probe ILMN_1795429; transcript variant 2, microarray element probe ILMN_2413527) genes were selected for differential expression and correlation analyses. These results were further confirmed by low-density qRT-PCR array.

## Low-density qRT-PCR-array (LDA)

The quantitative real-time polymerase chain reaction (qRT-PCR) was performed with TaqMan low-density array (LDAs; Applied Biosystems) according to the manufacturer's instructions. The functionality of TaqMan assays, the optimal amounts of RNA in cDNA synthesis and optimal amount of cDNA in qRT-PCR were first optimized for functional range and validated for inhibition using several concentrations in separate TaqMan assays. Sufficient RNA was available for 19 out of 24 LITAs (79.2%) and 64 out of 68 plaque (94.2%) samples. 60 (30 cases and 30 controls) out of 96 blood samples (62.5%) were selected for analysis based on pairwise matching according to BMI, age, gender and smoking status. In detail, 500 ng of total RNA per sample was transcribed to cDNA using the High Capacity cDNA Kit (Applied Biosystems). For the qPCR, LDAs were loaded with 7 µl cDNA synthesis product (175 ng of RNA), 43 µl $H_2O$ and 50 µl PCR Universal Master Mix (Applied Biosystems). The array contained technical triplicates. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, assayID Hs99999905_m1) was used as housekeeping gene control. The qRT-PCR data was analyzed with Expression suite software (Applied biosystems) using the 2-ΔΔCT method.

## Hierarchical Clustering and Correlation Analyses

*TLN1*, *TLN2*, *VCL* and well-characterized biomarkers of inflammation (cluster of differentiation 68 (CD68) and arachidonate 5-lipoxygenase (ALOX5)), and SMCs markers (calponin 1 (CNN1), smoothelin (SMTN)) [31] were used in the hierarchical clustering analysis to assess whether subgroups of samples had similar marker profiles. Furthermore, similarity of the expression levels (*TLN1*, *TLN2* and *VCL*) across the samples was studied. The procedure was performed using the heatmap.2 function from the gplots R library on all 68 artery samples. Pearson dissimilarity and average linkage were used for the hierarchical clustering of both genes and samples. To further investigate the *TLN1*, *TLN2* and *VCL* genes as measures of plaque cell composition, correlation analyses were performed using previously established macrophage and SMC-rich plaque signatures [31].

## Confocal Immunofluorescence Study of Frozen and Paraffin-Embedded Samples

For immunofluorescence labeling of frozen tissue sections, vascular samples from LITA and from atherosclerotic carotid artery were embedded into TissueTek O.C.T compound (Sakura Finetek, USA), frozen in liquid nitrogen and stored at −80°C. Leica CM 3050S (Leica Biosystems, Nussloch, Germany) cryostat was used to cut 6 µm sections of the frozen tissues. Before antibody staining, the tissue sections were air-dried at room temperature for 20 minutes and fixed with acetone at -20°C for 10 minutes. Fixed samples were air-dried for 15 minutes at room temperature, immersed into PBS (pH 7.4) and transferred to Shandon Sequenza (Thermo Shandon Ltd, Runcorn, UK) immunostaining cassettes. Nonspecific antibody binding was blocked by preincubating the tissue sections in blocking buffer containing 1% BSA and 0.3% Triton-X100 diluted in PBS (pH 7.4). All antibodies were diluted into the blocking buffer. The following primary antibodies were used to detect adhesion proteins and vascular cell markers in adjacent sections: mouse-anti-human PECAM-1 antibody (CD31, clone JC70A, Dako Agilent Technologies, Glostrup, Denmark) diluted 1:20 was used as a marker of endothelial cells, rabbit-anti-human Tal1 (clone ab71333, Abcam, Cambridge, UK) diluted 1:80 was used for talin-1, mouse-anti-human Tal2, (clone 68E7, Cancer Research Technology, London, UK) diluted 1:100 was used for talin-2 and rabbit anti human Vin (clone ab61186, Abcam, Cambridge, UK) diluted 1:50 was used vinculin. Samples were incubated with diluted primary antibodies at +4°C overnight, followed by washing 3 times with PBS. AlexaFluor-568 labeled goat-anti-mouse IgG (Cat # A11004, Thermo Fisher Scientific) and AlexaFluor-488 labeled donkey-anti-rabbit IgG (Cat # A21206, Thermo Fisher Scientific) antibodies diluted 1:100 were incubated on the samples for 1 hour at room temperature to detect the bound primary antibodies. Immunostained samples were washed 5 times with PBS, followed by one wash with deionized water. Glass coverslips were mounted on the samples by using Prolong Diamond (Cat # P36962, Thermo Fisher Scientific) containing DAPI for nuclear staining.

For immunofluorescence staining of formalin-fixed and paraffin-embedded (FFPE) samples, 4 µm sections were cut from paraffin-embedded samples of LITA and healthy carotid artery and abdominal aorta. Samples of LITA were collected from patients diagnosed with atherosclerosis, while the samples of carotid artery and abdominal aorta were collected from a cadaver with no coronary artery disease. Hematoxylin-eosin (HE) stained tissue sections were used to confirm normal tissue morphology of these samples. All tissue sections were deparaffinized and rehydrated by incubating them in xylene and in 99%, 95%, 70% and 50% ethanol solution for 10 min in each. For antigen retrieval, tissue sections were boiled in 10 mM sodium citrate buffer (pH 6) with 0.05% Tween-20 for 20 min in a microwave oven and allowed to slowly cool back to room temperature. Samples were washed 3 times with PBS (pH 7.4) and treated with 0.1% Sudan Black B for 20 min at room temperature to quench tissue autofluorescence. Samples were washed 3 times 10 min with PBS and immunostained using the same methods used for the frozen tissue sections. Antibody specificity was confirmed by using control samples with no primary antibodies.

The immunolabeled sections were examined under a laser scanning confocal microscope (Zeiss Cell Observer.Z1 equipped with a 63x/NA 1.4 oil immersion objective and Zeiss LSM780 confocal unit, Carl Zeiss Microscopy, Jena, Germany). For fluorophore excitation, 480 nm argon laser and 405 nm and 561 nm diode lasers were used together with suitable filter sets. For comparative analysis, the laser intensities, PMT gains and other settings were kept constant for all samples. Serial plane images were collected throughout the whole thickness of the sample at 200 nm intervals. During image processing, maximum intensity projections were used to extract high intensity areas from the image stacks into single images.

## Statistical Analyses

Statistical analyses were performed using R version 2.15.0. To estimate fold changes between groups in microarray analysis (MA), differences between medians (in log2 scale) were calculated. The log ratios were back-transformed to fold changes. To ease the interpretation, fold-change values <1 were

replaced by the negative of its inverse. Statistical significance of differences in gene expression was assessed using the nonparametric Wilcoxon signed-rank test and the log-transformed data. For associations between *TLN1*, *TLN2*, *meta-VCL* and *VCL* expression levels and SMC-rich plaque/macrophage markers, the Spearman correlation coefficient was used. To test the effect of covariates on expression levels of *TLN1*, *TLN2*, *meta-VCL* and *VCL*, Wilcoxon rank-sum test and Spearman correlation were used. The associations between covariates and expression levels were tested in the atherosclerotic plaque and LITA samples separately and were considered significant when $p<0.05/42$ to 0.001 according to the Bonferroni correction for multiple testing. Differences were considered significant when $p<0.05$.

### Limitations of the study

Due to the poor availability of control arterial samples from healthy persons with no coronary artery disease, LITA samples obtained during coronary artery bypass surgery were used as controls for the studied plaque samples. LITA samples were collected from patients diagnosed with coronary artery disease. Therefore, the levels of talin-1, talin-2 and vinculin transcript expression profiles in LITA may not exactly match their expression profile in artery samples from healthy subjects with no diagnosed CAD. Therefore, immunostaining of talin-1 was used to confirm similar expression pattern of talin-1 in the LITA samples from CAD patients and in artery sample from healthy subject (Figure 4). In addition, similar artery morphology for these samples was confirmed by observing tissue sections with HE-staining (Figure 4). Another limitation of our study is the relatively small sample group size used. The small sample group size results from the poor availability of suitable patient samples and it was taken into consideration in the interpretation of the results.

## Results

### Characteristics of the subjects and studied samples

The demographics and risk factors of studied population are presented in Table 1. All internal arteries used as controls were verified microscopically as normal. Body mass index, occurrence of hypercholesterolemia, high blood pressure, coronary artery disease and history of myocardial infarction differed significantly between control group and group with atherosclerotic plaques. For mononuclear and whole blood analysis, patients with coronary artery disease considered as case group differed significantly in hypercholesterolemia occurrence, statin medication use and history of myocardial infarction from the control group. Because of these significant differences in the population, the gene expression was analyzed separately between these groups.

*Table 1 Demographics and risk factors of the study patients included in the Tampere Vascular Study* [10].

| | Arterial | Plaque | Mononuclear/whole blood | |
|---|---|---|---|---|
| | Control | Case | CAD Control | CAD Case |
| No. of subjects | 24 | 68 | 44 | 52 |
| Age, y (median, SD) | 69.0 (8.6) | 70.0 (10.4) | 57.0 (8.6) | 56.5 (8.6) |
| Men (%) | 82.1 | 67,6 | 63.0 | 61.5 |
| Body mass index, kg/m$^2$ (median, SD) | 28.2 (5.1) | 26.0 (4.0)* | 27.7 (4.2) | 26.9 (4.3) |
| History of smoking, % | 64.3 | 75.0 | 53.3 | 65.4 |
| Diabetes mellitus, % | 32.1 | 23.5 | 8.7 | 19.2 |
| Hypercholesterolemia, % | 85.7 | 67.6* | 52.2 | 76.9* |
| Hypertension, % | 100.0 | 82.4* | 84.8 | 96.2 |
| Antihypertensive medication, % | 92.9 | 80.9 | 80.4 | 92.3 |
| Statin medication, % | 82.1 | 73.5 | 20.4 | 73.1* |

| | | | | |
|---|---|---|---|---|
| Coronary artery disease, % | 100.0 | 29.4** | 0.0 | 100.0*** |
| Myocardial infarction, % | 40.7 | 13.2* | 23.9 | 57.7*** |

Pearson chi-square test and Wilcoxon signed-rank test was used for categorical and continuous risk factors, respectively. $p<0.05^{*}$, $p<0.01^{**}$, $p<0.001^{***}$.

## Talin-1, talin-2 and vinculin transcripts are robustly expressed in left internal thoracic artery (LITA) controls with significant reduction in plaques.

The gene expression profiles of talin-1, talin-2 and vinculin were investigated to understand the molecular mechanisms behind atherosclerosis. All three transcripts were robustly expressed in LITA samples (Supplementary Table S1a) and in all plaque samples of the three analyzed arterial beds (Supplementary Table S1b). Microarray analysis (MA) and low-density qRT-PCR (LDA) array showed that all tested talin and vinculin transcripts were downregulated in atherosclerotic plaques (fc < -1.6, for all plaques, with MA and LDA, $p < 0.00001$ (Supplementary Table S2, Figure 1a-d). In more detail, all transcripts were downregulated in plaques of carotid, abdominal aortic and femoral arterial beds in comparison to LITA (Supplementary Table S2, Figure 1e-h). The downregulation of talin-1 and talin-2 was most substantial in femoral plaques in comparison to LITA, whilst meta-vinculin was most downregulated in carotid plaques and vinculin in abdominal aortic plaques in comparison to LITA.

[Figure 1]

## Expression of talin-1, talin-2 and vinculin transcripts is downregulated in both stable and unstable atherosclerotic plaques

We further characterized the expression profiles as a function of disease progression. According to microarray analysis, all tested transcripts were downregulated in all plaques in comparison with LITA controls (Supplementary Table S2, Figure 1a-d). Even though no significant reduction was observed between the stable and unstable atherosclerotic plaque, a negative tendency in expression was seen for talin-1, talin-2 and meta-vinculin between the stable and unstable plaques (Figure 1a-d).

## Talin-1 and vinculin transcripts are expressed in whole blood and circulating monocytes.

In order to evaluate if the expression profiles could be monitored using blood samples, whole blood and circulating monocytes were analyzed. Talin-1, meta-vinculin and vinculin were robustly expressed in whole blood samples while talin-2 was not detected in whole blood (Supplementary Table S1a). No significant difference was observed between patients with history of coronary artery disease (CAD) and controls in the whole blood expression levels of talin-1, talin-2, meta-vinculin and vinculin in MA (fold change (fc) = -1.01 – 1.03, for all, $p > 0.3$) or in LDA analysis (fold change (fc) = -1.13 – 1.05, for all, $p > 0.3$). Moreover, no significant differences were observed in the whole blood samples of the patients with hypercholesterolemia, statin usage or patients with myocardial infarction. Expression in circulating monocytes was statistically insignificant between controls and CAD patients for talin-1, talin-2, meta-vinculin or vinculin transcripts. Nominally significantly reduced expression was however observed for patients with myocardial infarction events for talin-1 (fc = -1.13, $p = 0.026$) and for vinculin (fc = -1.12, $p = 0.03$) in circulating monocytes. However, no associations between gene expression and clinical risk factors remained statistically significant after correcting for multiple testing.

## Hierarchical Clustering Analysis; association of talin-1, talin-2 and vinculin transcripts with SMC and inflammation markers

Hierarchical clustering based on the expression of the two inflammatory and two SMC markers showed distinct separation of plaque samples from the LITA controls. Expression of talin-1, talin-2 and vinculin was dependent on expression of SMC markers (*CNN1* and *SMTN*). Furthermore, high expression of *TLN1*, *TLN2*, *VCL* and SMC markers with low expression of inflammatory biomarkers *CD68* and *ALOX5* was observed in LITA controls (Figure 2). Also plaque samples of all tested arterial

beds separated into two distinct branches of the dendrogram. The samples exhibiting greater reduction in *TLN1*, *TLN2* and *VCL* expression contained mainly carotid arterial samples, whereas majority of femoral arterial beds showed smaller changes in the expression reduction.

[Figure 2]

## Talin-1, talin-2 and vinculin transcripts expression levels correlate with SMC-rich atherosclerotic plaque signature

To study the connection between gene expression and plaque composition, the expression profiles were correlated with the known markers of smooth muscle cells (SMC). Utilizing markers of M1 and M2 macrophages and SMC-rich plaque signature, we found that talin-1, talin-2 and both vinculin transcripts correlate positively with SMC-rich plaque signature and in majority negatively with M1 and M2 macrophage signatures (Supplementary Figure S1).

## Talin-1, talin-2 and vinculin localization in the atherosclerotic plaque cells according to Confocal Immunofluorescence Analysis

To get information about the protein localization within the blood vessel samples, immunofluorescence staining of frozen tissue sections from atherosclerotic plaque and LITA controls were used to study adhesion protein localization and expression in advanced disease. Talin-1 was found to be highly expressed in the vascular endothelial cells of LITA samples (Figure 3a). On the contrary, talin-1 staining was not observed in the endothelial cells of an atherosclerotic artery. Furthermore, in the thickened tunica intima (I) underlying the endothelial cells, gradual increase in talin-1 expression was seen towards tunica media (M).

Antibody staining for talin-2 was observed to only partially colocalize with talin-1 staining in the tunica intima (I) and tunica media (M) in LITA samples (Figure 3b). Interestingly, in tunica media of LITA controls, talin-2 expression was higher in the luminal side and gradually decreased towards tunica adventitia (A). In plaque samples, talin-2 expression in the endothelial cells was decreased, but not to the same extent as talin-1 expression. In LITA samples, vinculin was found to be expressed in both tunica intima (I) and tunica media (M), but not in tunica adventitia (A).

In tunica media, vinculin expression was higher close to the lumen and lower deeper inside the vessel wall (Figure 3c). In plaque samples, decreased expression of vinculin in endothelial cells was observed, but in the lower parts of the thickened intima vinculin was still expressed. As expected, the expression of endothelial adhesion molecule PECAM-1 was found to be higher in the endothelium of atherosclerotic plaques compared to the control samples from healthy arteries [32] (Figure 3a, c).

[Figure 3]

## Samples from LITA and healthy carotid artery and abdominal aorta show similar tissue morphology and talin-1 expression patterns

To confirm similar tissue morphology and talin-1 expression patterns in healthy carotid artery and abdominal aorta and in the LITA samples used as a control in this study, tissue sections of formalin-fixed and paraffin-embedded (FFPE) samples were stained with talin-1 antibody and hematoxylin-eosin (HE) staining. Immunofluorescence staining of FFPE samples with talin-1 antibody showed strong specific staining of talin-1 in tunica intima (I) and tunica media (M) in samples from LITA and from healthy carotid artery and abdominal aorta (Figure 4). Similarly to the sections from frozen LITA, the analyzed FFPE sections from LITA or healthy arteries showed uniform talin-1 staining

intensity, suggesting that the talin-1 gradient observed in plaque samples is unique feature. In addition, HE staining of the FFPE samples showed similar tissue morphology for samples from LITA and carotid artery and abdominal aorta (Figure 4). These experiments confirm the feasibility of using LITA samples from CAD patients as negative controls for samples from atherosclerotic plaques when studying expression patterns of focal adhesion proteins.

[Figure 4]

## Discussion

In this study we show for the first time that the gene expression of talin-1, talin-2, meta-vinculin and vinculin is significantly reduced in atherosclerotic plaques. Significant downregulation of expression was observed in all of the studied carotid, abdominal aortic and femoral arterial beds compared to LITA controls. However, expression of neither gene was changed in circulating monocytes or in whole blood samples in CAD patients compared to controls.

We speculate that reduction in talin-1 expression in the endothelium may be one of the initial triggers for the atherosclerotic plaque formation (Figure 5). Such downregulation could be caused by an external factor such as altered shear stress and increased blood pressure. It has long been suspected that the mechanosensing and mechanotransduction affects the DNA packing and may contribute to changes in the protein expression in health and in disease [33] [34]. Another reason may lie in the mutation affected control of gene expression levels leading to changes in the cell mechanobiology and susceptibility to plaque development. In addition, recently the importance of miRNAs in the regulation of gene expression in endothelial dysfunction has become evident, as discussed by Novák et al. [35]. Furthermore, one of the contributing factors of detected downregulation in this study may be the changed cell composition in the advanced atherosclerotic plaque which can be seen on the confocal images. Changes in acting mechanical force may also alter the signaling pathways related to focal adhesions and affect the expression levels. In connection to atherosclerosis, the magnitude of shear stress was shown to affect the expression of adhesion molecules facilitating endothelial cell-leukocyte adhesion at the vascular lumen (vascular cell adhesion molecule (VCAM), intercellular cell adhesion molecule (ICAM) or platelet endothelial cell adhesion molecule (PECAM) [36] [32]. Our previous studies have also shown that the focal adhesions are compromised by reduced expression of integrin family proteins and kindlin-2 in the endothelium and SMCs in the atherosclerotic plaque (*ITGA1*, *ITGAV*, *ITGB1*, *ITGB3*, *ITGB5*, *FERMT2*), while the leukocyte adhesion is accelerated by increased expression of leukocyte integrin-B2 and kindlin-3 [10]. The exact reasons for the observed reduction of the talin and vinculin expression level remain, however, unclear and require attention in future studies.

[Figure 5]

Altered expression of talin-1, 2, meta-vinculin and vinculin may have severe impact on the cell's ability to withstand varying magnitudes of acting mechanical forces, affect cell locomotion, cell/cell and cell-ECM communication since both talin and vinculin are among the major constituents of the focal adhesion complexes and are essential for cellular well-being [37]. Talin acts as molecular scaffolding protein and may contribute to adhesion signaling via its binding partners, converting mechanical signals to chemical cues [38]. Therefore, a reduction in talin-1 expression could render the endothelium and the vascular wall prone to endothelial injury compared to mechanically stable endothelial cell [39]. Endothelial injury triggers leukocyte adhesion and promotes inflammatory response at the intima due to exposure of subendothelial collagen and other ECM components. Confocal microscopy images of atherosclerotic plaque show increased PECAM-1 staining in the intima, which points to increased leukocyte adhesion molecules and progression of inflammation at the intima.

In addition, endothelium functions as a barrier for large molecules to enter the vessel wall and trigger pathological processes in the inner vessel layers [40] [41]. In other words, the mechanostability of endothelial cells including the endothelial intercellular gap and tight junction maintenance is crucial for healthy vessel wall. Experiments with talin knockout cells show dramatically decreased capability of cells to adhere, revealing the central role of talin in mediating the intracellular-extracellular connection. The reduction in talin-1, talin-2, meta-vinculin and vinculin expression in the endothelium may affect the ability of endothelial cells to adhere to each other and to ECM to form consistent blood-tissue barrier. This could allow the inflammatory agents to progress into the tunica media and trigger macrophage accumulation in the intima and media leading to arterial wall thickening and plaque formation. Such effect can be observed in the presented tissue images where plaque staining shows dramatically thickened intima layer with gross disorganization of the cells and low talin-1 and talin-2 intensity (reduced expression of talin-1 and talin-2 in plaque sample).

Studies by others have also shown the importance of talin-1 and talin-2 for cell, tissue and organ development [42] [43]. Such importance of talin proteins on development was illustrated by talin-1 knockout experiments in HUVEC cells [44] [45] leading to severe phenotypes in mice embryos. The phenotype is represented by abnormal vascular development affecting the growth of other major tissues [45]. Furthermore, increased talin-1 expression has been detected in aggressive cancers as well as in hypertrophic myocardium and failing heart [46] [47] [48]. Even though the downregulation of neither talin-1 nor talin-2 has been to date directly linked to or associated with a disease, the reduced expression may affect the vessel tissue formation, remodeling or healing and recovery. The confocal images of the atherosclerotic plaque show gross disorganization of the cells in the vessel wall. Such additional effects may lead into worsening of the site inflammation and progress of the disease to severe, unstable atherosclerotic plaques. Even though the difference in expression between stable and unstable atherosclerotic plaque reported here was not significant for the investigated transcripts, negative tendency in expression levels was observed for talin-1, talin-2 and meta-vinculin. This suggests that the expression of these genes is further reduced in the advanced disease.

The expression of talin-1 and talin-2 may also differ among the cells depending on the position in the vessel wall layer. As can be seen in the talin-2 LITA staining, the intensity of talin-2 expression in smooth muscle is higher in the layers closer to the intima where the mechanical impacts are expected higher. Similar stratification has not been observed for talin-1 or vinculin in LITA controls. Whether such stratification in healthy tissue is biologically and physiologically important remains open for further investigation.

## Conclusion

Talin-1, talin-2, meta-vinculin and vinculin expression is downregulated in atherosclerotic plaque. Downregulation of expression was observed in plaques from all of the studied peripheral arterial beds (carotid, abdominal aortic and femoral).

Even though the difference between stable and unstable atherosclerotic plaque was not significant for the investigated transcripts, negative tendency was observed for talin-1, talin-2 and meta-vinculin. This suggests that the expression of talins and vinculin is further reduced in the progression of the disease.

The central role of talin in cell adhesion proposes that the disintegration of the tissue in atherosclerosis could be partially driven by downregulation of talin, leading to loosening of cell-ECM interactions and reorganization of the tissue.

In conclusion we state that proteins contributing to mechanosensing, talin and vinculin, may have important role in atherosclerotic plaque formation and disease progression.

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## Figure Legends

Figure 1 Expression of talin (*TLN*) and vinculin (*VCL*) transcripts. Results of Microarray Analysis (MA). (a-d) Expression levels in LITA controls and all, stable and unstable atherosclerotic plaques. (e-f) Expression levels in carotid, abdominal aortic and femoral arterial bed compared to LITA controls.

Figure 2 Heat maps of log2 expression values. Talin-1 (*TLN1*), Talin-2 (*TLN2*) and vinculin (*VCL*) co-expression with biomarkers of inflammation (*CD68*, *ALOX5*) and smooth muscle cells markers (*CNN1*, *SMTN*). The expression values of each row (gene) are scaled to z-scores, color-code for expression values and arterial site is presented in the top-right corner. The dendrogram illustrates hierarchical clustering based on the seven robustly expressed genes. The top bar represents the arterial site origin of the sample tissue.

Figure 3 Tissue localization of talin-1, talin-2 and vinculin in atherosclerotic plaque from carotid artery and left internal thoracic (LITA) control. (a) talin-1 and PECAM-1, (b) talin-1 and talin-2, (c) vinculin and PECAM-1.Talin-1 was expressed in the endothelial cells of LITA, while talin-1 expression in plaque endothelial cells was not observed (a). Talin-2 expression was decreased in plaque endothelium as compared to LITA, but not as strongly as talin-1 expression (b). In LITA, high vinculin expression was observed in tunica intima and at the lumenal side of tunica media. In plaque samples, decreased vinculin expression was observed in the thickened tunica intima (c). PECAM-1 expression was found to be increased in plaque endothelium (a, c). L: lumen, I: tunica intima, E1: internal elastic lamina, E2: external elastic lamina, M: tunica media, A: tunica adventitia.

Figure 4 Tissue morphology and localization of talin-1 in immunostained FFPE sections of LITA from patients with coronary artery disease and healthy abdominal aorta and carotid artery. Uniform talin-1 immunostaining was observed in tunica intima and tunica media in both immunostained LITA samples, as well as in the samples from healthy abdominal aorta and carotid artery. HE-staining showed normal tissue morphology for all of the analyzed samples. In the merged images, talin-1 immunostaining is shown as green and DAPI chromatin staining as blue. L: lumen, I: tunica intima, E1: internal elastic lamina, E2: external elastic lamina, M: tunica media, A: tunica adventitia.

Figure 5 Simplified speculative model for downregulation of talin expression in vascular endothelium as initial trigger of atherosclerotic plaque formation.

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665 Fig. 1

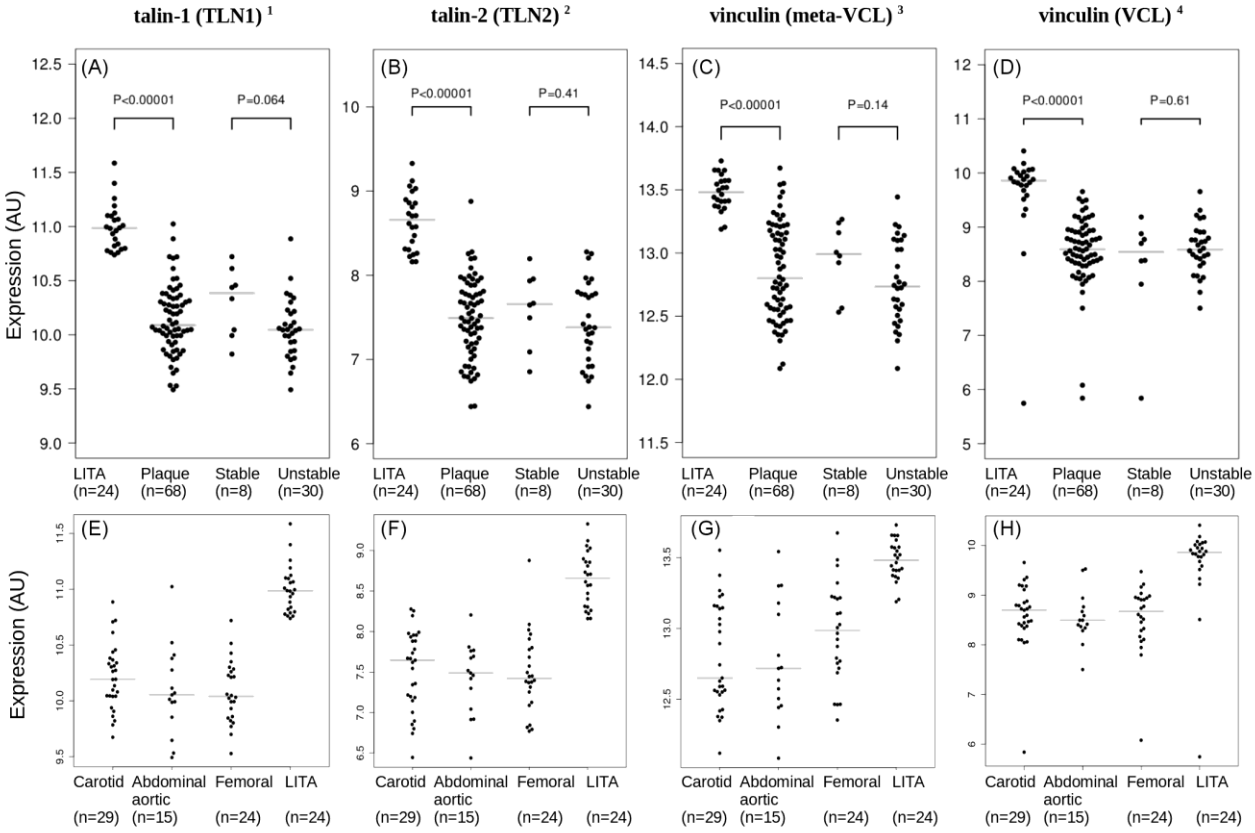


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Fig. 2

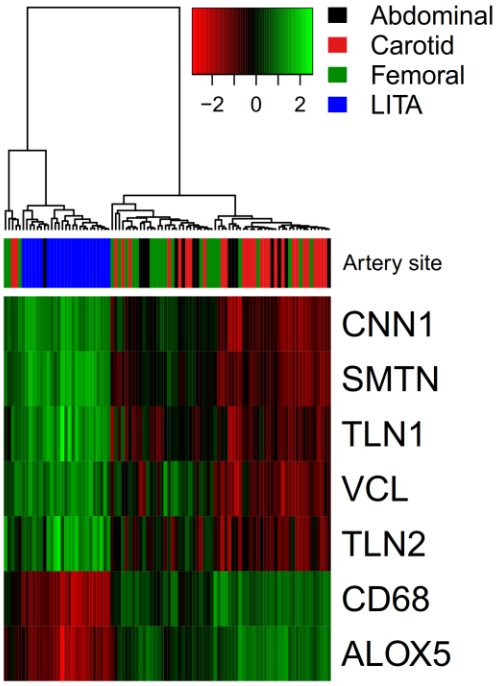
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Fig. 3

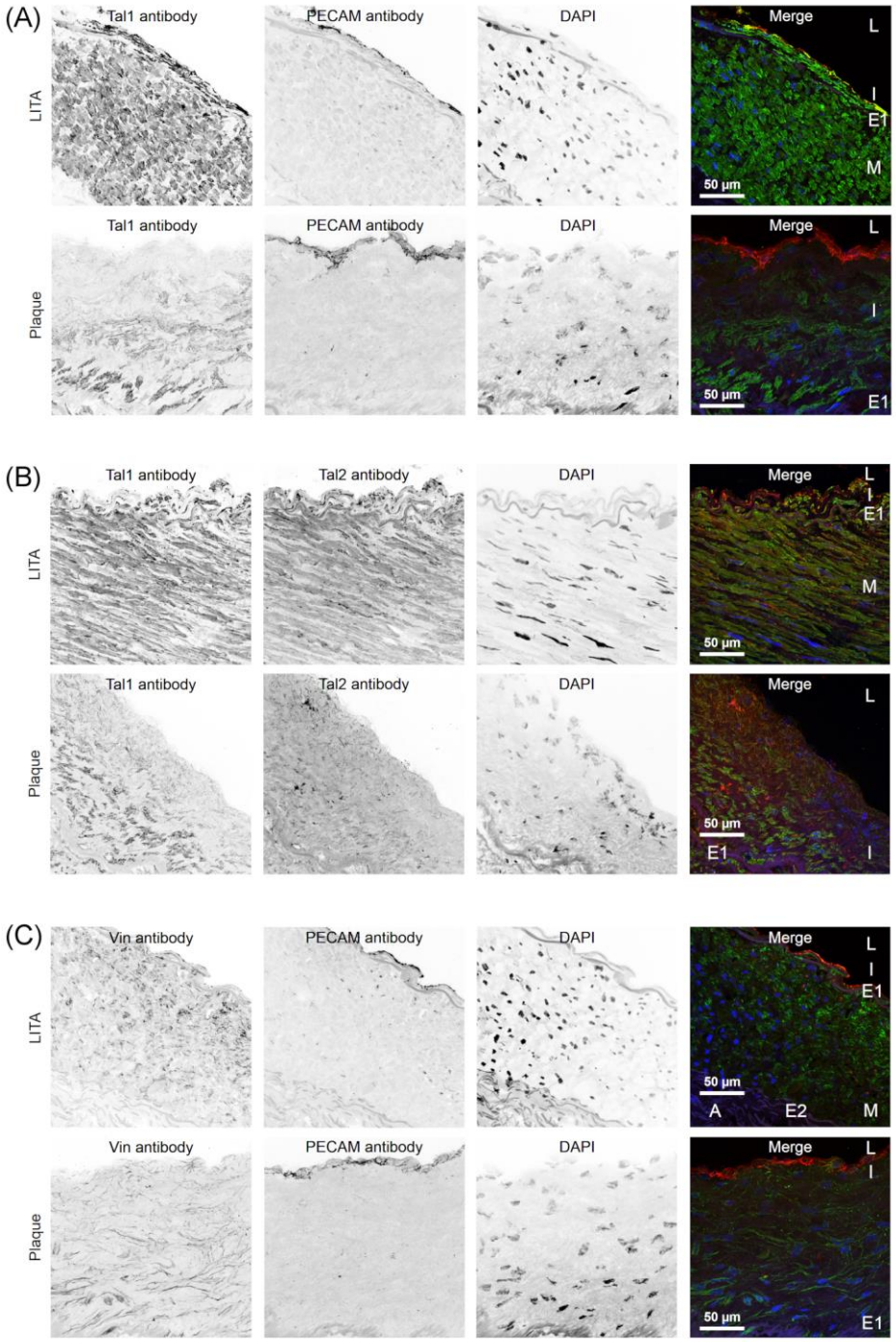
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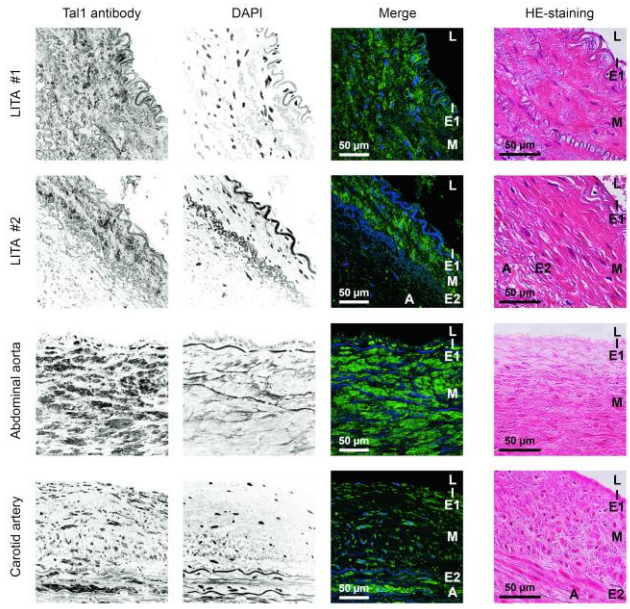
Fig. 4


Tal1 antibody
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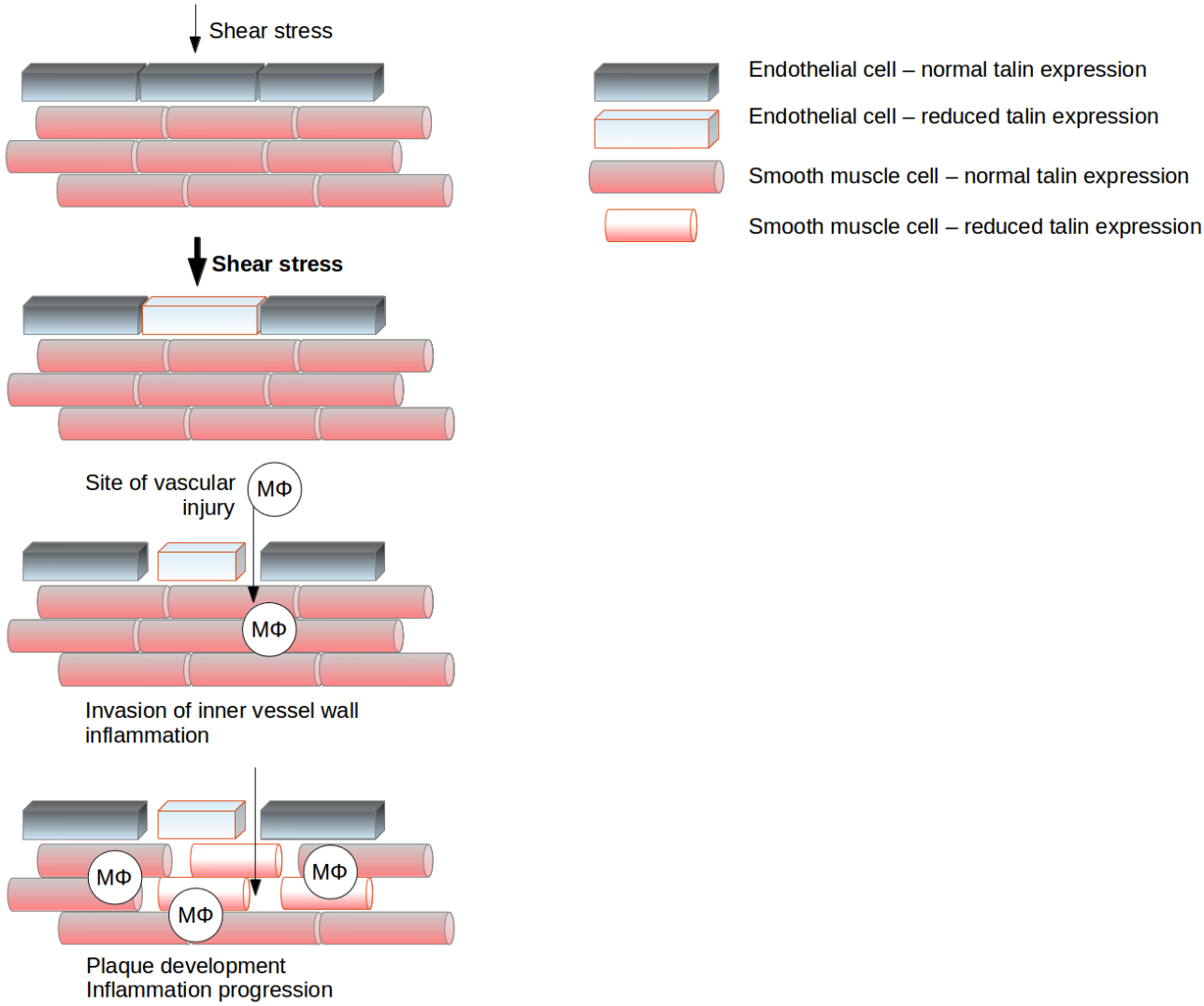
740 Fig. 5


Shear stress
Shear stress
Site of vascular injury
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Inflammation progression
Endothelial cell – normal talin expression
Endothelial cell – reduced talin expression
Smooth muscle cell – normal talin expression
Smooth muscle cell – reduced talin expression

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