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KINDLIN 3 (FERMT3) IS ASSOCIATED WITH UNSTABLE ATHEROSCLEROTIC PLAQUES, ANTI-INFLAMMATORY TYPE II MACROPHAGES AND UPREGULATION OF BETA-2 INTEGRINS IN ALL MAJOR ARTERIAL BEDS

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Short title: Kindlin-3 (FERMT3) in human atherosclerosis

Number of tables: 3; number of figures: 6

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Total word count: 5507

The Journal Subject Codes : Atherosclerosis : [142] Gene expression

ABSTRACT

BACKGROUND. Kindlins (FERMT) are cytoplasmic proteins required for integrin (ITG) activation, leukocyte transmigration, platelet aggregation and thrombosis. Characterization of kindlins and their association with atherosclerotic plaques in human(s) is lacking. **METHODS AND RESULTS**. Exploratory microarray analysis (MA) was first performed followed by selective quantitative validation of robustly expressed genes with qRT-PCR low-density array (LDA). In LDA, ITGA1 (1.30-fold, p=0.041) and ITGB3 (1.37-fold, p=0.036) were upregulated in whole blood samples of patients with coronary artery disease (CAD) compared to healthy controls. In arterial plaques, both robustly expressed transcript variants of FERMT3 (MA: 5.90- and 3.4-fold; LDA: 3.99-fold, p<0.0001 for all) and ITGB2 (MA: 4.81- and 4.92-fold; LDA: 5.29-fold, p<0.0001 for all) were upregulated while FERMT2 was downregulated (MA: -1.61-fold; LDA: -2.88-fold, p<0.0001 for both). The other integrins (ITGA1, ITGAV, ITGB3, ITGB5) were downregulated. All these results were replicated in at least one arterial bed. The latter FERMT3 transcript variant associated with unstable plaques (p=0.0004). FERMT3 correlated with M2 macrophage markers and in hierarchical cluster analysis clustered with inflammatory and macrophage markers, while FERMT2 correlated with SMC-rich plaque markers and clustered with SMC markers. In confocal immunofluorescence analysis, FERMT3 protein colocalized with abundant CD68-positive cells of monocytic origin in the atherosclerotic plaques, while co-localization of FERMT3 with HHF35 indicative of smooth muscle cells was low. **CONCLUSIONS**. Kindlin-3 (FERMT3) is upregulated in atherosclerotic, especially unstable plaques, mainly in cells of monocytic origin and of M2 type. Simultaneous upregulation of ITGB2 suggests a synergistic effect on leukocyte adherence and transmigration into the vessel wall.

Keywords: kindlin, integrin, immunohistochemistry, gene expression, atherosclerosis

INTRODUCTION

Endothelial dysfunction, infiltration of the arterial wall by macrophages (MΦ) and platelets, development of chronic inflammation, formation of foam cells, activation of smooth muscle cells (SMC), plaque neovascularization, hemorrhages and thrombosis are hallmarks of atherosclerosis [1,2]. Macrophage content is related with unstable, vulnerable plaques [3] which are prone to rupture and induce arterial thrombosis and cardiovascular events by activation of integrin-mediated platelet adhesion and aggregation [4]. Interestingly, oxidized low density lipoprotein [5], and especially monocytes activated by inflammation [6] induce integrin-mediated formation of monocyte-platelet aggregates. Platelets seem to have a critical role in leucocyte adhesion, transmigration into the vessel wall [7], promotion of inflammation [8] and promotion of foam cell formation [9]. These aggregates also modify the phenotype of monocytes, extravasation and foam cell formation and therefore promote atherosclerotic plaque progression [5]. All these events from the recruitment, extravasation and activation of MΦ, SMC and platelets utilize integrin-mediated mechanisms. For example, integrin $\alpha_{IIb}\beta_3$ mediates platelet aggregation by fibrinogen binding [10] and β_2 integrins Mac-1 (CD11b/CD18; $\alpha_m\beta_2$) and LFA-1 (CD11a/CD18; $\alpha_L\beta_2$) mediate macrophage binding [11]. Therefore, strategies to modulate integrin function are promising options in the current treatment of atherosclerosis involving interaction of cells with each other and with the extracellular matrix (ECM).

Kindlins are emerging molecules regulating integrin function and are ultimately required for integrin activation [12]. In contrast to the widely expressed kindlins-1 (FERMT1) and 2 (FERMT2), kindlin-3 (FERMT3) is restricted to hematopoietic cells and is particularly abundant in megakaryocytes and platelets [13,14]. Kindlin-3 (FERMT3) is also required for β_2

integrin-mediated leukocyte adhesion to endothelial cells as evidenced by kindlin-3 $(FERMT3)^{-/-}$ mice model [15]. Mutation in kindlin-1 (FERMT1) results in Kindler syndrome characterized by skin atrophy and ulcerative colitis [16] while there is no associated disease with kindlin-2 (FERMT2) mutation. Kindlin-3 (FERMT3) deficiency results in leukocyte adhesion deficiency (LAD type III) characterized by generalized hemorrhage, leukocyte adhesion deficiency, abnormal erythrocyte membrane organization and osteopetrosis, severe bleeding and resistance to arterial thrombosis [14]. Kindlin-3 (FERMT3) is also considered essential for integrin activation and platelet aggregation since platelets lacking kindlin-3 (FERMT3) cannot activate integrins despite normal talin expression [14]. Kindlin-3 (FERMT3) regulates activation of both $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$ [17], but the exact mechanism is not known (for review, see [18]).

Kindlins are important modulators of integrin mediated mechanisms, adherence of leukocytes to endothelium, transmigration in to the vessel wall and binding to extracellular matrix proteins, platelet activation and thrombosis, all of which are pivotal phenomena in atherosclerosis. The role of kindlins in human atherosclerosis in different arterial beds, plaque types and circulating cells is poorly understood. We hypothesized that kindlins are associated with human atherosclerosis and modify the plaque phenotype.

METHODS

Vascular samples

Vascular sample series from Tampere Vascular Study (TVS) [19,20,21,22,23,24] including femoral arteries, carotid arteries and abdominal aortas were obtained during open vascular procedures during 2005-2009 from patients fulfilling the following inclusion criteria: 1) carotid endarterectomy due to asymptomatic or symptomatic and hemodynamically significant (>70%) carotid stenosis, or 2) femoral or 3) aortic endarterectomy with aortoiliac or aortobifemoral bypass due to symptomatic peripheral arterial disease. The left internal thoracic artery (LITA) samples serving as controls were obtained during coronary artery bypass surgery (CABG) due to symptomatic coronary artery disease (CAD) and/or previous myocardial infarction (MI). An exclusion criterion was a patient's denial to participate in the study. Gene expression was analysed from carotid (n=29), abdominal aortic (n=15) plaques and femoral (n=24) plaques (cases), and as controls, from atherosclerosis free LITAs (n=28). The study has been approved by the Ethics Committee of Tampere Hospital District. All clinical investigation was conducted according to declaration of Helsinki principles and the study subjects gave informed consent. The samples were taken from patients subjected to open vascular surgical procedures in the Division of Vascular Surgery and Heart Centre, Tampere University Hospital. The vascular samples were classified according to recommendation of American Heart Association (AHA) [25]. The type V and VI atherosclerotic lesions were further histologically classified as stable and unstable according the presence of fissure/rupture, hemorrhage and/or thrombosis.

Whole blood and circulating mononuclear cell fractions

Tampere Vascular Study (TVS) whole blood and mononuclear cell collections were performed in 2008. The angiographically verified patient samples were pre-selected from a larger population based study [26]. RNA was isolated from the whole blood and mononuclear cells of individuals with angiographically verified CAD (n=52) and without coronary artery lesions (n=44). The participant pool comprises patients who have undergone an exercise stress test at Tampere University Hospital. The study is a cross-sectional study where after the exercise stress tests, the patients were treated according to Finnish Current Care Guidelines. The study began in the beginning of October 2001 with the goal of enrolling up to 5,000 patients. Data on the patients included were collected between October 2001 and the end of December 2004. Patient history data are based on hospital records and patient interviews. These data cover demographics—for example age, sex, weight, etc.—and lifestyle information, including alcohol consumption and physical exercise. Classical cardiovascular risk factors and symptoms are also extensively covered. The study also has access to the laboratory tests performed on the patients at Tampere University Hospital between October 1998 and the end of the study.

RNA isolation and microarrays (MA)

The fresh arterial tissue samples were soaked in RNALater solution (Ambion Inc., Austin, TX, USA) and isolated with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and the RNAEasy Kit with DNase Set (Qiagen, Valencia, CA, USA). From the whole blood fraction, the RNA was isolated with PAXgene tubes (BD, Franklin Lakes, NJ, USA) 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 the samples were stored in –80°C. The expression levels of arterial and whole blood samples were analyzed with an Illumina HumanHT-12 v3 Expression BeadChip (Illumina, San Diego, CA, USA) analyzing 47,000 transcripts of all known genes, gene candidates and splice variants. The microarray experiments for the mononuclear cell RNA were performed by using Sentrix® Human-6 Expression BeadChips analyzing over 46,000 transcripts (Illumina). Both arrays were run according to given instructions by the manufacturer and scanned with the Illumina iScan system. More detailed descriptions of the methodology and qRT-PCR validation of the microarrays have been published previously [27,28].

Microarray data analysis

After background subtraction, raw intensity data was exported using the Illumina GenomeStudio software. Raw expression data were imported into R (http://www.r-project.org/), log2 transformed and normalized by the LOESS normalization method implemented in the R/Bioconductor package lumi (www.bioconductor.org). LOESS normalization was selected for the data from all three tissues because it gave the best accuracy in comparison to qRT-PCR data for artery samples [27] (data not shown). Data quality control criteria included detection of outlier arrays based on the low number of robustly expressed genes and hierarchical clustering. Artery samples (n=92: 68 plaque, 24 LITA), peripheral blood samples (n=96: 52 CAD, 44 controls) and mononuclear cell samples (n=96: 52 CAD, 44 controls) fulfilled all data quality control criteria. The 68 atherosclerotic plaque samples included tissue from 29 cases with carotid artery, 15 cases with abdominal aorta and 24 cases with femoral artery. Probes were considered robustly expressed if the detection P value was <0.05 for at least half of the samples in the data set. Robustly

expressed genes were selected for further differential expression and correlation analyses and further confirmed by LDA analysis (below).

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 and the optimal amounts of RNA and cDNA with our samples have been studied by an individual assay. Both cDNA synthesis and qRT-PCR reaction have been validated for inhibition. 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. Shortly, 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 175 ng of cDNA, 43 µl H2O 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 analysed with Expression suite software (Applied biosystems) using the $2^{-\Delta\Delta C_T}$ method.

Hierarchical clustering and correlation analyses

Kindlin-2 (FERMT2) and 3 (FERMT3), well-characterized biomarkers of inflammation (CCL2, CD68, ALOX5) [29] and established markers of SMCs (CNN1, SMTN, MYH11) were used in the hierarchical clustering analysis to assess whether subgroups of samples had

similar marker profiles as well as whether Kindlin-3 (FERMT3) showed similar expression levels across the samples. The procedure was carried out using the heatmap.2 function from the gplots R library using all 92 arterial samples. Standard settings (Euclidean distance, complete linkage) were used for the hierarchical clustering of both genes and samples. To further investigate whether kindlin expression is a measure of plaque cell composition, correlation analyses were performed using previously established magrophage/plaque signatures [30].

Confocal immunofluorescence (IF) study

For immunofluorescence labelling, vascular samples from femoral arteries, carotid arteries and LITAs from three different patients were fixed in paraformaldehyde, transferred to 30% sucrose buffer, embedded in tissue O.C.T. (Tissue Tek II, Lab-Tek Products), frozen in liquid nitrogen and then stored at −80°C until use. Kindlin-3 (FERMT3) was detected with rabbit polyclonal anti-kindlin-3 (FERMT3) antibody, ab68040 (Abcam, Cambridge, MA, USA). The following primary antibodies were used to detect vascular cell markers in adjacent sections: CD68 (mouse anti-human CD68, clone PG-M1, DakoCytomation, Glostrup, Denmark) was used as marker of monocytes and macrophages and HHF35 as a marker for muscle actin (mouse anti-human muscle actin, clone HHF35, DakoCytomation). All antibodies were diluted in PBS containing 1% BSA and 0.3% of Triton X-100. Frozen sections (7 µm) were cut with Microm HM 560 (Thermo Scientific, Waltham, USA) and thaw-mounted onto Superfrost Plus (Menzel-Gläser, Braunschweig, Germany). After that, glass slides were let to stay 2 hours inside the cryotome. The sections were incubated overnight with the mixture of kindlin-3 (FERMT3) antibody (diluted 1:100) and CD68 antibody (diluted 1:10) or HHF35 antibody (diluted 1:10) followed by a mixture of fluorescein-conjugated sheep anti-mouse

antibody (1:10, Amersham Biosciences, Freiburg, Germany) and DyLight549 conjugated goat anti-rabbit antibody (1:100, AbD Serotec, Kidlington, UK) for 30 min at 37ºC. Sections were mounted in a mixture of glycerol and PBS (3:1) containing 0.1% paraphenylenediamine. The immunolabelled sections were examined under a confocal spinning disk microscope (Wallac-Perkin Elmer Ultraview) attached to a Nikon Eclipse Ti microscope. Images were acquired using double laser excitation at 488 nm (fluorescein) and 561 nm (rhodamine) and recorded with Andor EMCCD camera (Andor Technology, Belfast, Northern Ireland). Serial plane images were collected throughout the whole thickness of the fluorescent preparation. 2D images were obtained by projection of serial tomographs. For comparative analysis, all sections were scanned at the same magnification and confocal laser microscope settings.

Statistical analyses

Statistical analyses were performed using R version 3.0.1 (http://www.r-project.org/). Fold changes were calculated between medians and statistical significance of differences was assessed using the non-parametric Wilcoxon signed-rank test. For associations between kindlin-2 (FERMT2), and 3 (FERMT3) levels and SMC/macrophage markers, the Spearman correlation coefficient was used. Differences were considered significant when $p<0.05$. For subgroup analyses of the effect of demographics or significant risk factors on gene expression, significance was defined as fold-change >1.5, $p<0.01$.

RESULTS

Characteristics of the subjects and studied vascular, whole blood and mononuclear cell samples

The description of study population is presented in Table 1. Of the atherosclerotic samples, 74.6% were classified as type V-VI advanced plaques. All the internal mammary arteries that were used as control were microscopically verified as normal. Body mass index (BMI), the frequency of hypercholesterolemia, hypertension, CAD and myocardial infarction differed between arterial plaques and controls. Therefore, separate analyses addressing the effects of these covariates on gene expression results were conducted. In patients with mononuclear and whole blood samples, the frequency of hypercholesterolemia, statin usage, CAD, and myocardial infarction differed between cases and controls (Table 1). The effect of these covariates on gene expression was therefore subsequently analysed.

Table 1. Demographics and risk factors of study patients.

	Arterial control	**Arterial plaque**	**Mononuclear/ whole blood control**	**Mononuclear/whole blood case**
Number of subjects	24	68	44	52
Age, years (median, SD)	69.0 (9.6)	70.0 (10.4)	57.0 (8.6)	56.5 (8.6)
Men (%)	82.1	67.6	63.0	61.5
BMI, 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 (%)	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 user (%)	82.1	73.5	20.4	73.1*
CAD (%)	100.0	29.4**	0.0	100.0***
MI (%)	40.7	13.2*	23.9	57.7***

Pearson chi-square test p<0.05*, p<0.01**, p<0.001***

β3 integrin but not kindlins are upregulated in whole blood in coronary atherosclerosis

According to exploratory microarray analysis (MA), among kindlins, only kindlin-3 (FERMT3) was robustly expressed in whole blood samples and of the integrin transcripts, ITGAV, ITGB1, ITGB2 and ITGB3 were robustly expressed in whole blood samples (Table 2). These transcripts were further studied using LDA-analysis, in which expression of kindlin-3 (FERMT3) did not differ in whole blood samples of patients with CAD compared with healthy controls (1.08-fold, p=0.451). However, according to LDA, ITGB3 (1.37-fold, p=0.036) was upregulated in patients with CAD. Hypercholesterolemia, statin usage, CAD, or MI (Table 1) were not associated with the expression levels of kindlins 1,2 or 3 (FERMT1,2,3) or integrins.

Table 2. Kindlin (FERMT1,2,3) and integrin transcripts expressed in whole blood samples, left internal thoracic artery controls (LITA) and/or arterial plaques.

	Whole Blood (N=96)			**LITA (N=24)**			**Plaque (N=68)**			**Accession code**
Symbol	**N (p<0.05)**	**%**	**>50%**	**N**	**%**	**>50%**	**N**	**%**	**>50%**	
FERMT1	0	0	0	4	16.7	0	0	0	0	NM_017671.4/ILMN_1696585
FERMT2	1	1.04	0	24	100	1	68	100	1	NM_006832.1/ILMN_1695290
FERMT3	96	100	1	24	100	1	68	100	1	NM_031471.4/ILMN_2366330
FERMT3	96	98.5	1	17	70.8	1	67	98.5	1	NM_031471.4/ILMN_2366334
ITGA1	2	2.08	0	24	100	1	68	100	1	NM_181501.1/ILMN_1802411
ITGAV	92	95.8	1	24	100	1	68	100	1	NM_002210.2/ILMN_2169439
ITGB1	96	100	1	24	100	1	68	100	1	NM_002211.2/ILMN_1723467
ITGB1	96	100	1	24	100	1	68	100	1	NM_033669.1/ILMN_2383934

ITGB1	3	3.13	0	0	0	0	1	1.5	0	NM_133376.1/ILMN_1784454
ITGB1	1	1.04	0	0	0	0	0	0	0	NM_033668.1/ILMN_1754233
ITGB2	96	100	1	24	100	1	68	100	1	NM_000211.2/ILMN_2175912
ITGB2	96	100	1	24	100	1	68	100	1	NM_000211.1/ILMN_1654396
ITGB3	90	93.8	1	20	83.3	1	52	76.5	1	NM_000212.2/ILMN_1733324
ITGB5	96	100	1	24	100	1	68	100	1	NM_002213.3/ILMN_1668374
ITGB5	2	2.08	0	2	8.3	0	0	0	0	NM_944676.1/ILMN_1701619
ITGB6	0	0	0	0	0	0	0	0	0	NM_000888.3/ILMN_1789846

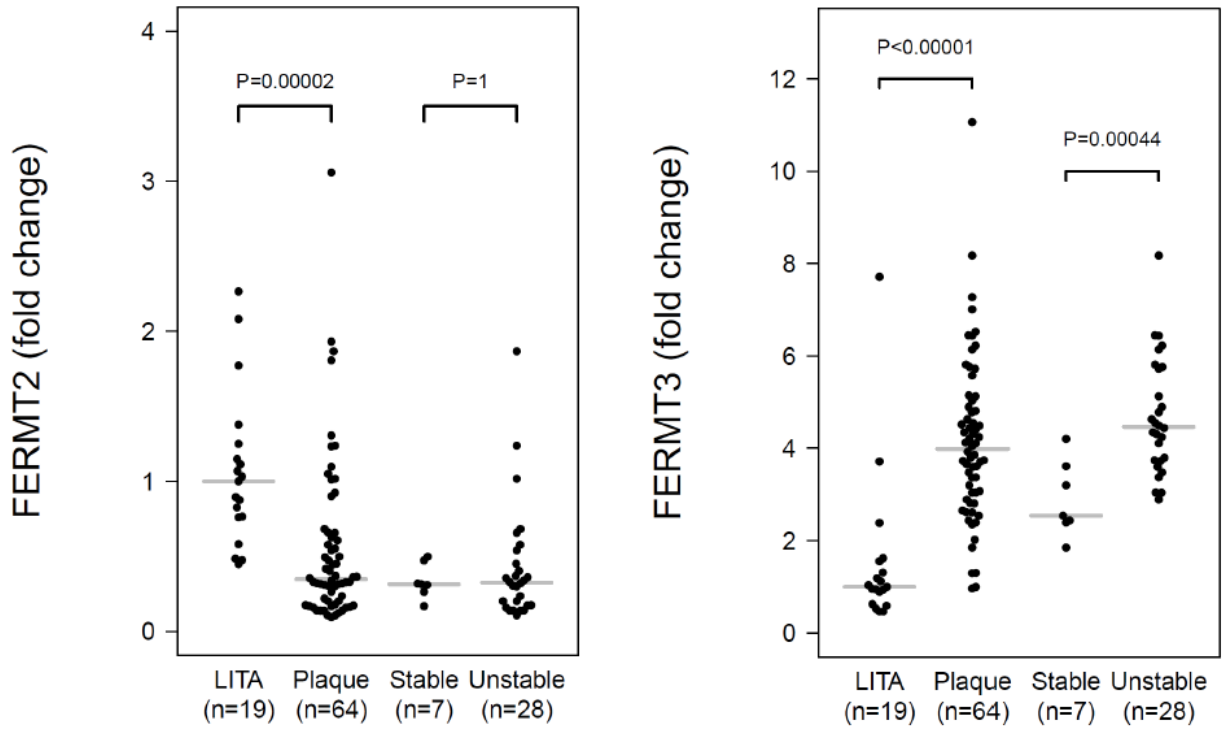
	Carotid (N=29)			Aorta (N=15)			Femoral (N=24)			Accession code
Symbol	N (p<0.05)	%	>50%	N	%	>50%	N	%	>50%	
FERMT1	0	0	0	0	0	0	0	0	0	NM_017671.4/ILMN_1696585
FERMT2	29	100	1	15	100	1	24	100	1	NM_006832.1/ILMN_1695290
FERMT3	29	100	1	15	100	1	24	100	1	NM_031471.4/ILMN_2366330
FERMT3	29	100	1	15	100	1	23	95.8	1	NM_031471.4/ILMN_2366334
ITGA1	29	100	1	15	100	1	24	100	1	NM_181501.1/ILMN_1802411
ITGAV	29	100	1	15	100	1	24	100	1	NM_002210.2/ILMN_2169439
ITGB1	29	100	1	15	100	1	24	100	1	NM_002211.2/ILMN_1723467
ITGB1	29	100	1	15	100	1	24	100	1	NM_033669.1/ILMN_2383934
ITGB1	0	0	0	0	0	0	1	4.2	0	NM_133376.1/ILMN_1784454
ITGB1	0	0	0	0	0	0	0	0	0	NM_033668.1/ILMN_1754233
ITGB2	29	100	1	15	100	1	24	100	1	NM_000211.2/ILMN_2175912
ITGB2	29	100	1	15	100	1	24	100	1	NM_000211.1/ILMN_1654396
ITGB3	25	86.2	1	7	46.7	0	20	83.3	1	NM_000212.2/ILMN_1733324
ITGB5	29	100	1	15	100	1	24	100	1	NM_002213.3/ILMN_1668374
ITGB5	0	0	0	0	0	0	0	0	0	NM_944676.1/ILMN_1701619
ITGB6	0	0	0	0	0	0	0	0	0	NM_000888.3/ILMN_1789846

Kindlin-3 (FERMT3) is upregulated and correlates with plaque instability with simultaneous upregulation of β2 integrin and downregulation of other integrins while kindlin-2 (FERMT2) is downregulated in atherosclerotic plaques

According to MA analysis, in arterial plaques and in all analysed arterial bed locations, only kindlin-2 (FERMT2) and 3 (FERMT3) were robustly expressed, while kindlin-1 (FERMT1) was not in any of them (Table 2). In atherosclerotic plaques in comparison with healthy controls kindlin-2 (FERMT2) was downregulated (MA: -1.61-fold, $p<0.0001$; LDA: -2.88-fold, $p<0.0001$) while both transcript variants of kindlin-3 (FERMT3) were upregulated (MA: 5.90-

and 3.4-fold, p<0.0001 for both; LDA: 3.99-fold, p<0.0001) (Figure 1, supplementary figure 1). Kindlin-3 (FERMT3) was also associated with unstable plaques (p=0.0004) (Figure 1).

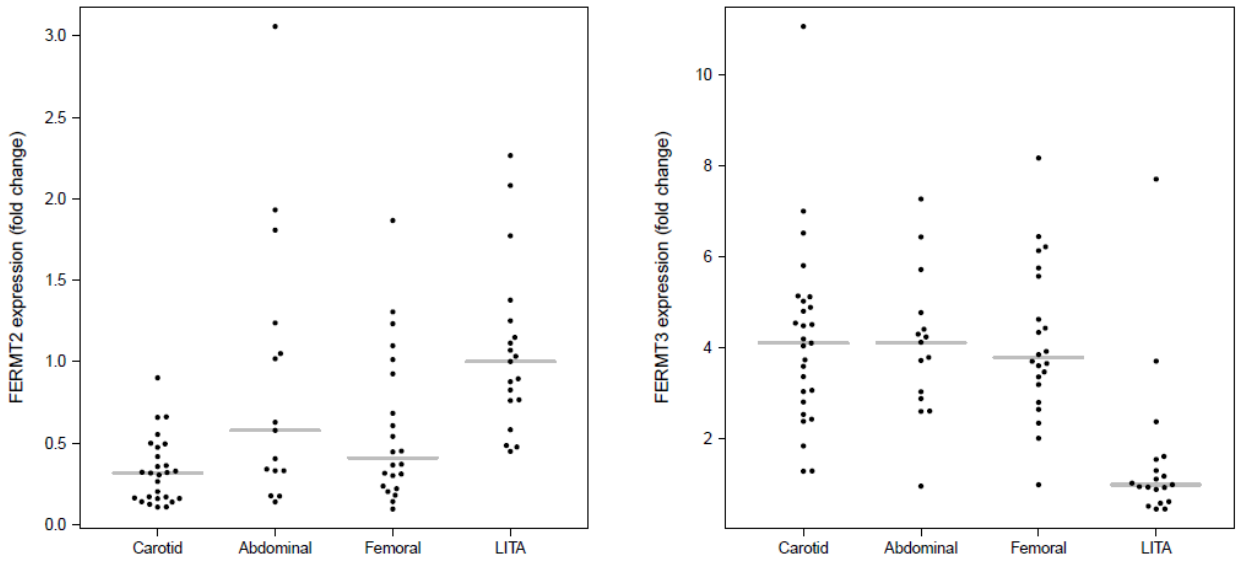
Figure 1. Gene expression changes of kindlin-2 (FERMT2) and kindlin-3 (FERMT3) in human atherosclerotic plaques and control samples (left internal thoracic artery [LITA]) as analyzed by qRT-PCR LDA assay. Dot-plots showing expression differences between the plaque and LITA samples and between stable and unstable plaque phenotypes in a subgroup of advanced plaques (stary V and VI). The horizontal line is the median expression value. For corresponding microarray results, see supplementary figure 1.



In arterial bed specific analysis, kindlin-2 (FERMT2) was downregulated (MA: -1.62-fold, p<0.0001; LDA: -3.17, p<0.0001) while kindlin-3 (FERMT3) transcript variants were upregulated (MA: 6.60- and 4.20-fold, p<0.0001 for both; LDA: 4.11-fold, p<0.0001) in carotid plaques in comparison to controls (Figure 2, supplementary figure 2). The femoral

plaques showed similar expression patterns of kindlin-2 (FERMT2) (MA: -1.61-fold, p<0.0001; LDA: -2.46-fold, p<0.010) and kindlin-3 (FERMT3) (MA: 3.30-fold, p<0.0001, LDA: 3.78, p<0.0001). In abdominal aortic plaques kindlin-2 (FERMT2), although robustly expressed, did not differ statistically according to LDA-analysis (MA: -1.57-fold, p<0.0001; LDA: -1.73-fold, p=0.099) while kindlin-3 (FERMT3) was upregulated (MA: 5.76-fold, p<0.0001; LDA: 4.13-fold, p<0.0001) (Figure 2, supplementary figure 2).

Figure 2. Gene expression changes of kindlin-2 (FERMT2) and kindlin-3 (FERMT3) in human atherosclerotic plaques according to arterial sites and control samples (left internal thoracic artery [LITA]) as analyzed by qRT-PCR LDA assay. Dot-plots showing expression differences between the plaques and LITA samples (stary V and VI). The horizontal line indicates the median expression value. For corresponding microarray results, see supplementary figure 2.



Of the integrins, ITGA1, ITGAV, ITGB1, ITGB2, ITGB3 and ITGB5 were robustly expressed in the arterial plaques and all plaque locations with the exception of ITGB3 in aortic plaques (Table 2).

Generally, both transcript variants of ITGB2 were upregulated while ITGA1, ITGAV, ITGB3, ITGB5 were downregulated in atherosclerotic plaques in comparison with healthy controls and also in carotid and aortic plaques in comparison to controls (Table 3). When analyzing different arterial sites, ITGB2 was upregulated and ITGA1 and ITGB5 were downregulated in all arterial sites (Table 3). ITGAV was downregulated also in carotid and aortic plaques in comparison to controls (Table 3). ITGB3 was downregulated in atherosclerotic plaques in comparison with healthy controls and also in carotid and femoral plaques in comparison to controls. BMI, the frequency of hypercholesterolemia, hypertension, CAD and MI (Table 1) were not associated with the expression levels of kindlins 1,2 or 3 (FERMT1,2,3) or integrins.

Table 3. Integrin transcripts robustly expressed in arterial plaques and left internal thoracic artery controls (LITA) analysed with microarray (MA) and low-density PCR array (LDA).

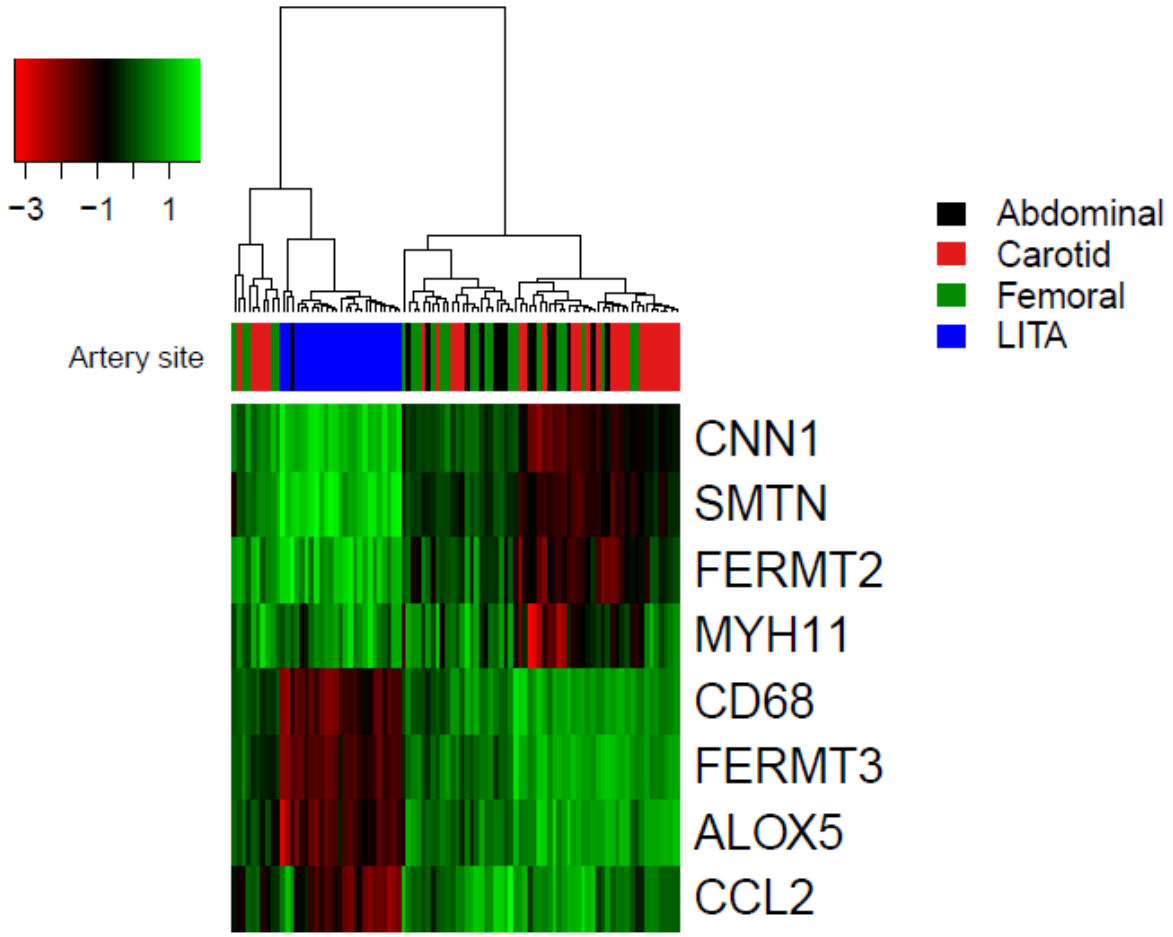
	Carotis		Aorta		Femoralis		All plaques	
Gene	MA	LDA	MA	LDA	MA	LDA	MA	LDA
ITGA1[1,a]	-1.21*	-2.59***	-1.64***	-4.00***	-1.03	-2.02***	-1.23*	-2.53***
ITGAV[2,b]	-1.16**	-1.36*	-1.01	-1.55*	-1.26	-1.26	-1.10*	-1.37*
ITGB1[3,c,d]	1.05/1.09	-1.62	1.13/-1.17	-1.40	1.09/-1.09	-1.41	1.09/-1.05	-1.49
ITGB2[4,e,f]	5.70***/5.60***	5.62***	4.42***/4.85***	5.88***	4.36***/4.46***	4.41***	4.81***/4.92***	5.29***
ITGB3[5,g]	-1.04	-2.15***	N.A.	N.A.	-1.03	-1.77**	-1.09	-2.17***
ITGB5[6,h,i]	1.07/-1.12*	-1.77**	-1.19*/-1.11*	-1.82**	-1.05/-1.12*	-1.66**	-1.01/-1.12	-1.76***

LDA gene (assay): [1]Hs00235006_m1; [2]Hs00233808_m1; [3-4]Hs00559595_m1; [4]Hs00164957_m1; [5]Hs01001469_m1; [6]Hs00174435_m1. Microarray accession code/probe id: [a]NM_181501.1/ILMN_1802411; [b]NM_002210.2/ ILMN_2169439; [c]NM_002211.2/ILMN_1723467; [d]NM_033669.1/ILMN_2383934; [e]NM_000211.2/ILMN_2175912; [f]NM_000211.1/ILMN_1654396; [g]NM_000212.2/ILMN_1733324; [h]NM_002213.3/ILMN_1668374; [i]NM_944676.1/ILMN_1701619

Hierarchical clustering analysis – the association of kindlin-3 (FERMT3) MΦ and kindlin-2 (FERMT2) with SMC markers

Hierarchical clustering based on the expression of the 6 inflammation and SMC marker genes showed distinct separation of plaque samples from controls (Figure 3). Furthermore, the plaque samples from the three arterial beds coalesced in two branches of the dendrogram. This clustering of kindlin-3 (FERMT3) was dependent on the expression of the MΦ markers but independent of the artery bed type (Figure 3). In contrast, clustering of kindlin-2 (FERMT2) was dependent on the expression of the SMC markers (Figure 3).

Figure 3. Heat maps of log2 expression values of kindlin-2 (FERMT2) and -3 (FERMT3) and biomarkers of inflammation and smooth muscle cells. Expression values for each row (gene) are scaled to z-scores and color-coded according to the legend on the left. The dendrogram depicts hierarchical clustering based on the 8 robustly expressed genes (CD68, CCL2, ALOX5, CNN1, SMTN, MYH11, FERMT2, FERMT3). The top bars indicate the arterial site of the sample tissue.



Kindlin-3 (FERMT3) expression levels are correlated with M2 signatures while kindlin-2 (FERMT2) expression correlates with SMC-rich plaque signature

Utilizing previously published markers of M1 and M2 macrophages and SMC-rich plaque signature, it was found that kindlin-2 (FERMT2) correlated positively with SMC-rich plaque signature (Figure 4) and mainly negatively with M2-macrophage-specific signature (Figure 5). In contrast, kindlin-3 (FERMT3) correlated negatively with SMC-rich plaque signature (Figure 4) and positively with M2-macrophage–specific signature (Figure 5).

Figure 4. Correlation among kindlin-2 (FERMT2) and -3 (FERMT3) mRNA levels and top 25 genes differentially expressed between regions of human atherosclerotic plaque enriched in smooth muscle-rich (SMC) plaque signature. Spearman correlation coefficient (r) and statistical significance values (P) are shown.

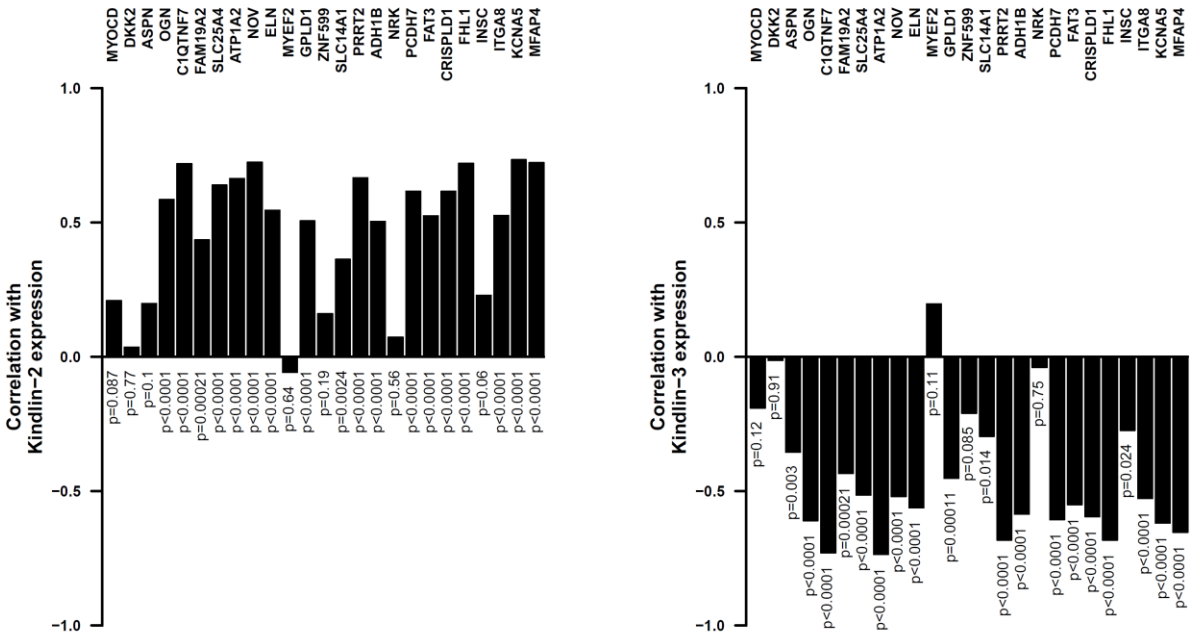
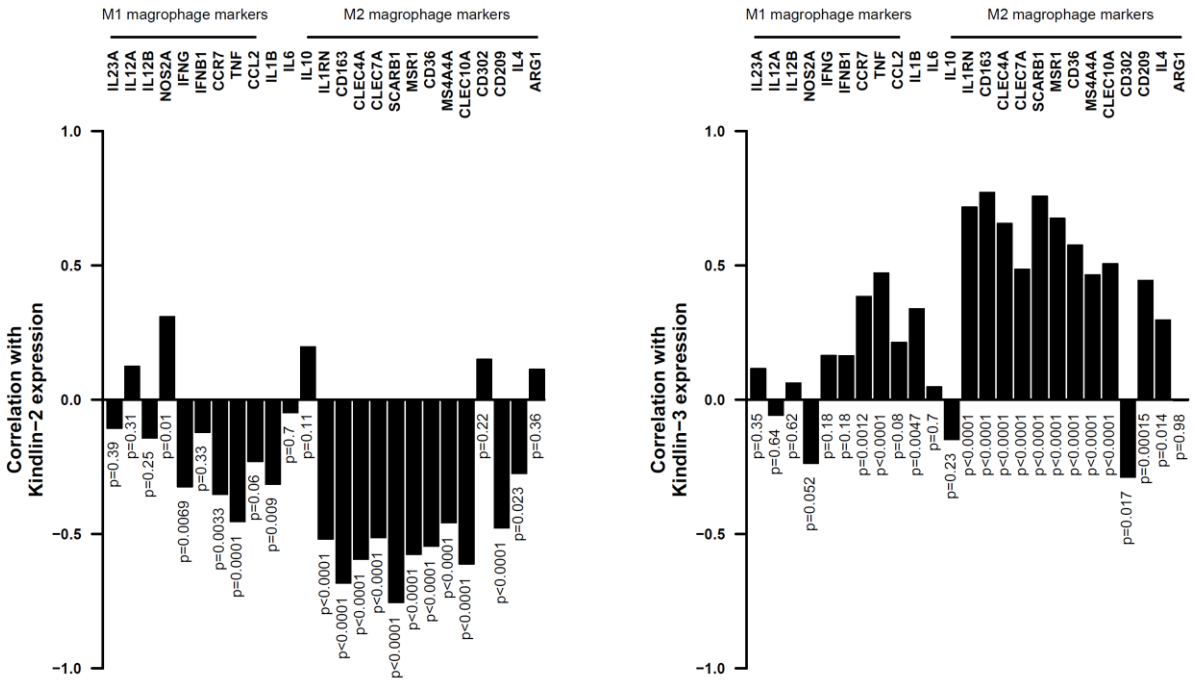


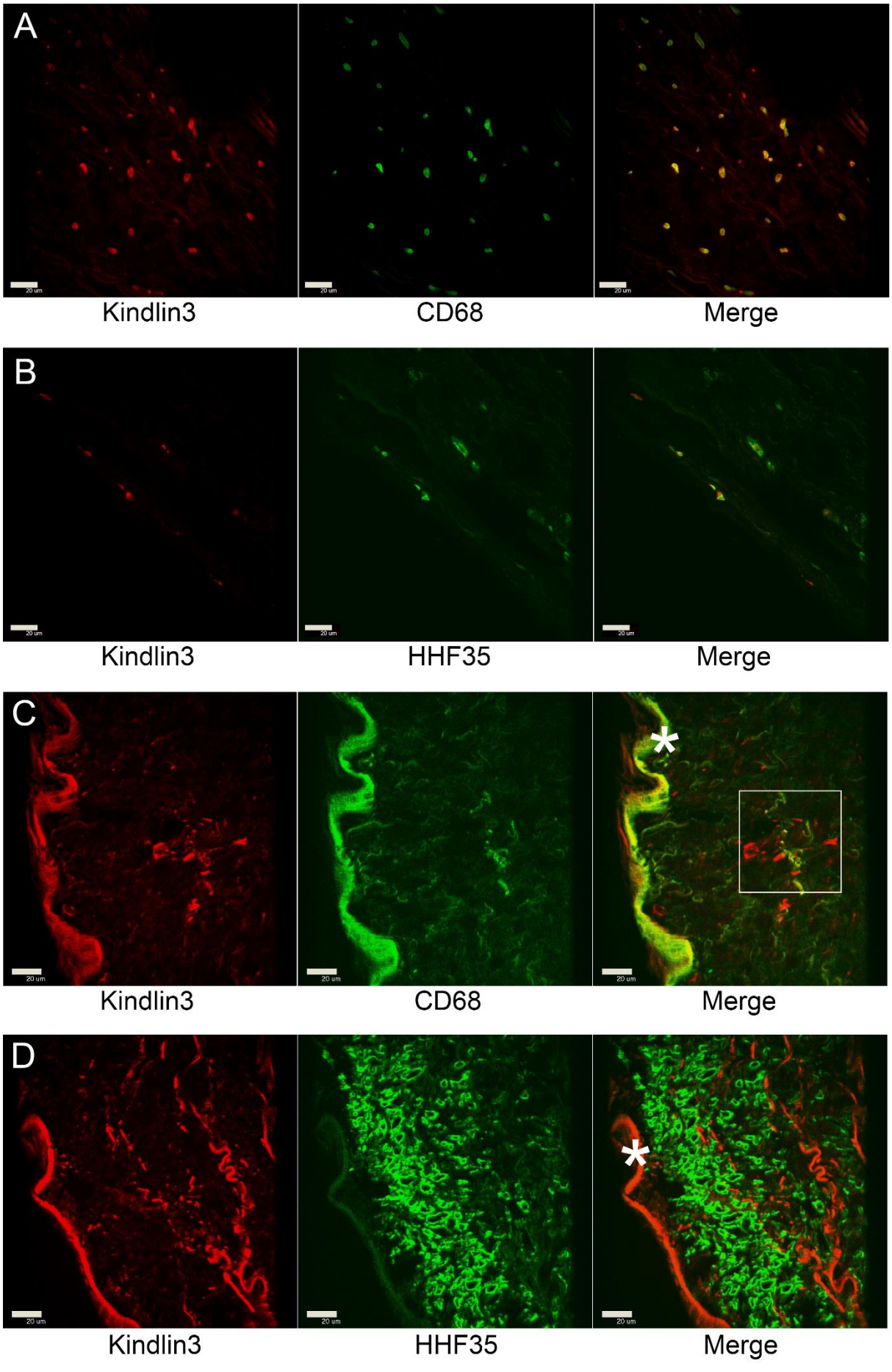
Figure 5. Correlation among kindlin-2 (FERMT2) and -3 (FERMT3) mRNA levels and established M1/M2 macrophage markers in human atherosclerotic plaque. Spearman correlation coefficient (r) and statistical significance values (P) are shown.



Confocal immunofluorescence analysis of kindlin-3 (FERMT3) localization in the atherosclerotic plaques

Due to significant upregulation of kindlin-3 (FERMT3) in the vessel wall, we aimed to study its co-localization with MΦ and SMC markers. In line with hierarchical clustering and correlation analyses, confocal immunofluorescence analysis showed that kindlin-3 (FERMT3) was co-localized with CD68 indicative of cells of monocytic origin in the atherosclerotic plaques (Figure 6). Only few inflammatory cells were detected in LITA controls, those showing kindlin-3 (FERMT3) expression (Figure 6C). Kindlin-3 (FERMT3) was not co-localized with HHF35, which is a marker of smooth muscle cells (Figure 6B, 6D). However, the number of smooth muscle cells was very low in the atherosclerotic plaques (Figure 6B), which demonstrates the dramatic changes in the structure of the diseased arterial walls.

Figure 6. Kindlin-3 (FERMT3) expression in CD-68 positive monocytes (A) and smooth HHF35-positive muscle cells (B) of atherosclerotic plaque and left internal thoracic artery (LITA) control (C, D). Kindlin-3 (FERMT3) is highly expressed in plaque macrophages and only in scant smooth muscle cells at the plasma membrane. In the immunofluorescent images, CD68-positive macrophages and HHF35-positive smooth muscle cells are shown in green and kindlin-3 (FERMT3) staining in red. Intense staining artifact at lamina elastica is indicated (*) and area of interest marked with a square.


A
Kindlin3
CD68
Merge
B
Kindlin3
HHF35
Merge
C
Kindlin3
CD68
Merge
D
Kindlin3
HHF35
Merge

DISCUSSION

Our present findings show for the first time that kindlin-3 (FERMT3) is upregulated in human atherosclerotic plaques, and is associated with severe, unstable atherosclerotic plaques mainly in cells of monocytic origin with anti-inflammatory M2-macrophage signatures. Synergistically, upregulation of integrins, especially β2 may indicate kindlin-3 (FERMT3) mediated inside-out activation of integrins as a mechanism behind the leukocyte adhesion and transmigration. In contrast, kindlin-2 (FERMT2) was downregulated in plaques and associated with SMC-rich plaque markers.

Since kindlins are essential molecules modifying integrin function and activation, they are a pre-requisite for several processes involved in atherogenesis, i.e. cellular adhesion to each other and to ECM, especially for adherence of leukocytes to endothelium, transmigration in to the vessel wall, platelet aggregation and thrombosis. Our observation of upregulation of integrins α1 and β3 in whole blood of patients with CAD supports the shift of the balance towards a pro-thrombotic and pro-atherogenic state. Direct binding of kindlin-3 (FERMT3) is required for platelet fibrinogen receptor $\alpha_{IIb}\beta_3$ activation and subsequent thrombosis [31] and atheroprogression [32]. According to previous demonstration of the effect of kindlin-3 (FERMT3) deficiency [14], one may speculate that overexpression of kindlin-3 (FERMT3) in the plaques might result in thrombosis and increased leukocyte adherence as occurs in generalized atherosclerosis. As kindlin-3 (FERMT3) is selectively expressed in cells of hematopoietic origin, it may serve as a potential target for the design of therapeutics aimed at specifically disrupting integrin activation in platelets.

Surprisingly, in contrast to whole blood samples, in arterial plaques, synergistic upregulated expression of kindlin-3 (FERMT3) and β_2 integrins suggests promotion of adherence of leukocytes, especially monocytes to endothelium and subsequent promotion of migration into the vessel wall since LFA-1 (i.e. $\alpha_L\beta_2$) and Mac-1 (i.e. $\alpha_m\beta_2$) are critical mediators of macrophage binding [11,33]. The significance of downregulation of kindlin-2 (FERMT2) and the other integrins (ITGA1, ITGAV, ITGB3, ITGB5) remains unclear. Since differential expression of kindlins did not occur either in whole blood or in circulating mononuclear cells, this indicates that possible alteration of kindlin expression in monocytes may occur during adherence and transmigration into the vessel wall but not in the freely circulating cells in the blood. The exact stimulus for this phenomenon is not known but kindlin-3 (FERMT3) may mediate both inside-out [12] and also outside-in signaling [34] resulting in activation of integrins. According to our results, overexpression of kindlin-3 (FERMT3) in arterial plaques is most probably due to high amount of macrophages and platelets in the plaque although the interaction of kindlin-3 (FERMT3) and beta integrins is also essential requirement for homing of T-lymphocytes [35] also critically involved in the atherosclerotic process. We could demonstrate colocalization of kindlin-3 (FERMT3) protein principally with abundant CD68-positive cells, which indicates cells of monocytic origin in the atherosclerotic plaques. Surprisingly, within arterial wall, according to correlation and hierarchical cluster analyses, kindlin-3 (FERMT3) associated with M2-macrophage signatures while kindlin-2 (FERMT2) associated with SMC-rich plaque markers. These findings suggest, that in atherosclerotic plaque kindlin-3 (FERMT3) might be involved in anti-inflammatory response since M2 macrophages secrete anti-inflammatory cytokines and are activated via an alternative pathway including cytokines secreted by T-helper cells of type 2 [36]. The negative correlation between kindlin-2 (FERMT2) and -3 (FERMT3) in the plaque samples likely reflects the different localization

of their expression within the plaques rather than any causal regulatory mechanism between the two kindlins in the same location. Our findings of association of kindlin-3 (FERMT3) with unstable plaques indicates that it may also be related with plaque remodellation since integrin signaling via kindlin-3 (FERMT3) is also required for osteoclast-mediated bone resorption and formation of podosomes required for this action [37]. Interestingly, our group and others have previously demonstrated the presence of large, multinucleated osteoclast-like cells (OCL) in advanced atherosclerotic plaques [23]. Therefore, it is tempting to speculate whether kindlin-3 (FERMT3) is operative in both anti-inflammatory and resorptive processes within the plaque. The role of downregulation of kindlin-2 (FERMT2) is unclear but it might be related with regulation of plaque neovascularization and vascular permeability (for review, see [38]). Previous studies have shown that Kindlin 3 is important for the function of macrophages, especially in their integrin activation, which regulates the adhesion of macrophages [37,14]. Since integrins are in central role in the differentiation of monocytes to macrophages [39], our findings suggest that kindlin-3 might be one of the key factors regulating the monocyte-macrophage differentiation process, thus leading to increase in the amount of M2 macrophages in atherosclerotic plaques.

As a potential limitation, it must be emphasized that due to due to ethical restrictions, we were not able to obtain any corresponding healthy arteries from carotid, aortic and femoral regions as controls. Obviously, this would allow more accurate analysis. The higher frequency of CAD and previous MI in arterial control patients is directly related to the fact that control patients were subjected to coronary artery revascularization due to CAD and/or MI. The higher frequency of hypercholesterolemia and hypertension in arterial control patients may be related to a potential diagnostic bias due to diagnostic exposure – patients

with peripheral arterial disease are more often directly referred by a primary care practitioner to a vascular surgeon while those with CAD are referred to a cardiologist and then to a thoracic surgeon. The effect of these differences in sample groups on gene expression were analysed and they did not have a statistically significant effect. Due to systemic nature of atherosclerosis, the internal thoracic artery samples of patients subjected to CABG may be affected by atherosclerosis and several humoral mediators, even though no microscopic evidence of plaques could be demonstrated. This could potentially alter the differences in gene expression compared to a situation where plaques are compared to arterial control samples from healthy subjects. Since the whole blood samples were obtained from different subjects, the gene expression values obtained from arterial tissues may not be directly compared to those found in whole blood or mononuclear cell samples.

In conclusion, our results implicate that upregulation of kindlin-3 (FERMT3) occurs in atherosclerotic plaques, especially in advanced unstable arterial plaques mainly in cells of monocytic origin and of M2 type. Simultaneous upregulation of integrins, especially β_2 indicate a synergistic effect on leukocyte adherence and transmigration into the vessel wall. In contrast to kindlin-3 (FERMT3), kindlin-2 (FERMT2) was downregulated in plaques and associated with SMC-rich plaque markers. These findings warrant future experiments aiming at therapeutical modulation of kindlins in models of experimental atherosclerosis.

ACKNOWLEDGEMENTS

This research is funded by European Union 7th Framework Program grant number 201668, Atheroremo. This study was supported with grants from the Medical Research Fund of Tampere University Hospital (grant X51001 for T.L. and X51410 for V.P.H), the Emil Aaltonen Foundation (T.L., N.OK), the Pirkanmaa Regional Fund of the Finnish Cultural Foundation, the Research Foundation of Orion Corporation, the Jenny and Antti Wihuri Foundation and the Academy of Finland (Grant nos. 104821, 136288, 273192 and 286284) and Yrjö Jahnsson Foundation (T.L.). Niku Oksala was supported by grants from the Finnish Angiology association, Maire Taponen foundation and Paavo Nurmi Foundation.

DISCLOSURES

None.

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