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Published in:
Atherosclerosis

DOI:
[10.1016/j.atherosclerosis.2015.06.023](https://doi.org/10.1016/j.atherosclerosis.2015.06.023)

2015

[Link to publication](#)

Citation for published version (APA):

Shami, A., Tengryd, C., Ascitutto, G., Bengtsson, E., Nilsson, J., Hultgårdh, A., & Goncalves, I. (2015). Expression of fibromodulin in carotid atherosclerotic plaques is associated with diabetes and cerebrovascular events. *Atherosclerosis*, 241(2), 701-708. <https://doi.org/10.1016/j.atherosclerosis.2015.06.023>

Total number of authors:
7

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Expression of fibromodulin in carotid atherosclerotic plaques is associated with diabetes and cerebrovascular events

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- Plaque formation and lipid retention are reduced in ApoE/fibromodulin-null mice
- We analyzed the content of human plaques obtained by carotid endarterectomy
- Fibromodulin and lumican correlated with plaque lipids and proinflammatory cytokines
- Fibromodulin was higher in symptomatic plaques and in plaques from diabetics
- Fibromodulin correlated with occurrence of post-operative cerebrovascular events

Expression of fibromodulin in carotid atherosclerotic plaques is associated with diabetes and cerebrovascular events

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Cover title: Fibromodulin and lumican in human atherosclerosis

Keywords: Fibromodulin, lumican, atherosclerosis, diabetes, vulnerable plaque

Word count: 4240 (excl. references)

Tables and figures: 2 tables, 3 figures

ABSTRACT

Aims The small leucine-rich proteoglycans fibromodulin and lumican are functionally related extracellular matrix proteins involved in the regulation of collagen fiber formation. Fibromodulin-deficient apolipoprotein E-null mice have decreased vascular retention of lipids and reduced development of atherosclerosis suggesting that fibromodulin may influence the disease process. The aim of the present study was to investigate if fibromodulin and lumican are expressed in human carotid plaques and to determine if their expression is associated with the occurrence of preoperative symptoms and with risk for postoperative cardiovascular events.

Methods and Results 153 plaques (51% symptomatic) obtained by carotid endarterectomy were included in this study. Plaque content was analyzed by immunohistochemistry and plaque cytokine content by multiplex technology. Fibromodulin and lumican were widely expressed in plaques and fibromodulin expression was significantly higher in symptomatic plaques. Expression of fibromodulin was significantly higher in plaques obtained from patients with diabetes and a high fibromodulin expression was associated with a higher incidence of post-operative cerebrovascular events, whereas no such associations were seen for lumican. Fibromodulin expression also correlated with plaque lipids and several pro-inflammatory cytokines. In addition, fibromodulin expression correlated with low levels of smooth muscle cells and the anti-inflammatory cytokine IL-10.

Conclusions These observations support previous experimental findings in mice for a role of fibromodulin in atherosclerosis and provide clinical evidence of the involvement of fibromodulin in the inflammatory processes that characterize atherosclerotic plaque vulnerability. They also suggest that this is of particular importance in diabetes.

INTRODUCTION

The extracellular matrix (ECM) is of key importance for maintaining the stability of atherosclerotic lesions.^{1, 2} Plaque rupture, due to degradation of the fibrous cap, is today considered to be the main cause of the development of acute myocardial infarction (MI) and stroke.^{3, 4} The ECM of atherosclerotic plaques is composed of a number of different proteins and glycoproteins, the most abundant being collagen, elastin and proteoglycans.^{5, 6} ECM components interact to maintain the mechanical stability of the plaque as well as perform several regulatory functions. Binding of LDL to vascular wall proteoglycans is also an initiating factor in plaque development⁷.

Fibromodulin is a 59 kDa proteoglycan that binds to collagen type I⁸ and its primary structure was determined in 1989 by Oldberg et al.⁹ This small leucine-rich repeat proteoglycan (SLRP) is involved in ECM remodeling by regulating collagen fiber assembly¹⁰ and by influencing collagen scaffold formation^{11, 12} by mechanisms that remain to be fully characterized. Fibromodulin shares close homology (~50%) with lumican, another member of the SLRP family.¹³⁻¹⁵ Like fibromodulin, lumican binds to collagen type I and contributes to collagen synthesis.¹¹

We have recently shown that plaque formation is reduced in ApoE-deficient mice lacking fibromodulin and that this is associated with an abnormal formation of collagen fibers in atherosclerotic plaques as well as with decreased lipid retention in the vascular ECM.¹⁶ Reduced lipid uptake was also observed in macrophages cultured on fibromodulin-deficient ECM.

The expression and role of fibromodulin and lumican in symptomatic human atherosclerotic plaques has not been previously investigated. Our previous results suggesting that fibromodulin is involved in plaque development by affecting ECM structure and lipid retention, together with previous publications discussing lumican expression in healthy and pathological vasculature,¹⁷⁻²¹ raise the question of possible contribution by these SLRPs to atherosclerotic plaque development and its stability. In the present study we have thus for the first time analyzed the expression of fibromodulin and lumican in human carotid plaques in relation to the occurrence of preoperative cerebrovascular symptoms (namely strokes, transient ischemic attacks (TIA) or amaurosis fugax) and postoperative cardiovascular (CV) events. We also assessed the association between the plaque expression of fibromodulin and lumican and the content of plaque lipids, inflammation and indicators of active remodeling.

MATERIALS AND METHODS

Additional Materials and Methods may be found in the online data supplement.

Patients

One hundred and fifty patients (out of which 3 were treated bilaterally) who underwent carotid endarterectomy (CEA) between 2005 and 2011 at the Vascular Department at Skåne University Hospital (Malmö, Sweden) were enrolled in this study after giving written informed consent. The study protocol was approved by the local Regional Ethical Committee (approval reference number 472/2005) and conformed to the principles of the Declaration of Helsinki. Clinical characteristics of the patients are described in Supplemental tables I and II in the online data supplement.

Postoperative events

The Swedish national inpatient health register was analyzed in order to identify postoperative CV events, with corresponding International Classification of Diseases, Tenth Revision (ICD-10) codes G45, G46, I20 to I25, I60 to I69 and I97 from January 1998 to December 2010. This is a nationwide validated register where more than 99 percent of all somatic (including surgery) and psychiatric hospital discharges are registered.²² In doubtful cases, information was gained through telephone interviews and medical chart reviews. All causes deaths were verified against the National Population Register. Definition of outcomes is accounted for in detail in the online data supplement.

Immunohistochemistry

Carotid plaque sections were immunohistochemically stained using primary antibodies against fibromodulin (kindly provided by D. Heinegård, Lund University, Sweden), lumican (ab168348; Abcam, Cambridge, UK), Glycophorin A (M0819, Dako Sweden, Stockholm, Sweden), collagen I and collagen III (ab6308 and ab6310 respectively; Abcam, Cambridge, UK). A biotinylated goat anti-rabbit IgG (Vector BA-1000, Vector Laboratories Inc, Burlingame, CA, USA) or rabbit F(ab')₂ anti-mouse IgG (ab98668, Abcam, Cambridge, UK) was used as secondary antibodies. In addition, immunohistochemical stains for smooth muscle α -actin and CD68) were performed as previously described.^{23, 24} Isotype control antibodies were used in concentrations corresponding to that of each primary antibody (ab27478 and ab81032 from rabbit and mouse, respectively; Abcam, Cambridge, UK) – representative images are shown in Supplementary Figure I. Immunoreactivity was quantified blindly using the imaging software program BioPix iQ version 2.3.1 (Biopix Ab, Gothenburg, Sweden). Residual media was not included during image analysis.

Homogenate analysis

Active Caspase 3 (cleaving at the Asp175/Ser176 site) was measured in carotid plaque homogenate using Human Caspase-3 ELISA (Invitrogen, Life Technologies, Carlsbad, CA). Cytokines were assessed in plaque homogenate (Human Cytokine/Chemokine Immunoassay, Millipore Corporation, MA, USA) and analyzed with Luminex 100 IS 2.3 (Austin, TX). MMPs (1-3, 7, 9, and 12) and tissue inhibitors of MMPs (TIMPs 1-3) were analyzed in plaque homogenate supernatants as previously described.²⁵ All analyses were performed according to the manufacturer's instructions and results were normalized to plaque wet weight.

Statistical Methods

Continuous variables were not normally distributed and are thus presented as median with interquartile range (IQR; 25th percentile to 75th percentile), while categorical variables are expressed as percentages. During immunohistochemistry and homogenate analyses Mann-Whitney U test and Spearman's rank correlation were used to assess correlations in continuous variables and Chi-square test in categorical variables. Freedom from postoperative events was calculated by life-tables according to Kaplan-Meier survival analysis. Correction for was done through Cox regression analysis. Age, gender, diabetes, hypertension, coronary heart disease, smoking and statin use were used as covariates in the regression model. A P-value of <0.05 was considered statistically significant. Statistical analysis was performed using SPSS 22.0 (IBM Corp., Armonk, NY, USA).

RESULTS

Plaque expression of fibromodulin and lumican

Expression of fibromodulin and lumican was found in the fibrous cap and shoulder regions, as well as in the core, though positive staining did not generally appear in all regions in the same plaque (Figure 1A-D). Fibromodulin and lumican immunoreactivity most often appeared in similar plaque regions when comparing staining patterns from consecutive sections. However, the extent of the positive stain often varied. Only in three plaques was fibromodulin and lumican expression consistently found in completely different regions. Finally, fibromodulin expression was generally absent from the outermost regions of plaques which represent the interface between the plaque and residual media while expression of lumican frequently occurred in this region.

When comparing with a Hematoxylin & Eosin (H&E) stain, plaque regions with positive fibromodulin and lumican immunoreactivity were found in both cellular and acellular regions. Using Masson's trichrome as an overview staining, positive immunoreactivity for fibromodulin and lumican were also detected in regions with a dense ECM such as the cap, as well as in ECM-poor regions such as the core (Supplementary Figure II). In the majority of the plaques fibromodulin and lumican was found in similar regions as lipids, as detected through Oil Red O staining and, to some extent, also as macrophages detected by the marker CD68. However, the CD68-staining was generally stronger and more extensive than the fibromodulin and lumican immunoreactivity, especially in the plaques from asymptomatic patients.

Immunohistochemical staining patterns for neither fibromodulin nor lumican matched with that of the marker smooth muscle α -actin, detecting differentiated (i.e. α -actin expressing and thus contractile) SMCs. However, a number of plaques (23% for both fibromodulin and lumican) contained small regions of corresponding positive immunoreactivity. We also found fibromodulin and lumican to partially localize to similar plaque regions as collagen types I and III (Supplementary Figures III and IV).

Fibromodulin and lumican are both associated with lipids and pro-inflammatory cytokines

Fibromodulin and lumican expression in the human plaques correlated positively with lipid content as assessed by Oil Red O staining and with the red blood cell maker Glycophorin A indicating intraplaque hemorrhage (Table 1). In addition, fibromodulin immunoreactivity correlated inversely with SMC content as assessed by staining for smooth muscle α -actin, while immunoreactivity for lumican was found to correlate with content of elastin and macrophages as detected by the marker CD68. Immunoreactivity for neither protein correlated with the presence of calcified plaque regions or overall fibrillar structures as assessed by Masson's trichrome staining. However, fibromodulin expression did correlate with collagen type I, but not type III, immunoreactivity, whereas no correlation was found between lumican expression and either type of collagen (Table I, Supplementary Figures III and IV). Finally, there was a correlation between fibromodulin and lumican expression levels.

There were significant associations between the plaque fibromodulin content and the expression of the pro-inflammatory cytokines MIP-1 β and sCD40L, as well as with the expression of VEGF

measured in plaque homogenates (Table 2). Additionally, fibromodulin correlated inversely with the levels of the anti-inflammatory cytokine IL-10. Lumican content correlated only with the pro-inflammatory cytokine RANTES. Positive correlations were also found between both fibromodulin and lumican and the active form of Caspase 3 ($r=0.215$, $p=0.015$ and $r=0.181$, $p=0.041$, respectively).

Finally, both fibromodulin and lumican expression were positively correlated to MMP1 and MMP9 measured in plaque homogenates (Supplementary table I). Moreover, fibromodulin expression was associated with the plaque content MMP12, while lumican expression was associated with MMP7. Positive correlations between both proteins and TIMP1 were also found.

Fibromodulin is associated with pre-operative symptoms

Fibromodulin content expressed as percentage of stained area was higher in plaques from patients with preoperative symptoms than in those from patients without symptoms (22.7% (7.7-32.8%), versus 9.6% (4.1-32.8%), $P<0.001$; Figure 1E). There was no significant difference in lumican expression in plaques from patients with and without symptoms (Figure 1F). Fibromodulin, but not lumican, expression was also found to be higher in plaques from patients with diabetes compared to non-diabetic patients (22.6% (10.0-34.3%) versus 9.6% (4.1-25.0%), $P=0.001$; Figure 2A-B). The median time interval between symptoms and CEA was 25 days (IQR 12-27 days, with a minimum of one day and a maximum of 120 days). There was no correlation between the level of fibromodulin or lumican and the time between the pre-operative symptom and operation. This suggests that there is no certain relation between these components and the repair of the plaque directly after rupture.

Among patients suffering from diabetes, fibromodulin content was higher in plaques from symptomatic patients compared to plaques from asymptomatic patients (30.3% (15.2-39.0%) versus 13.9% (4.4-23.2%), $P=0.015$, Figure 2C). Fibromodulin content was also significantly higher in symptomatic plaques from diabetics than in symptomatic plaques from non-diabetics (30.3% (15.2-39%) versus 13.1 (4.5-29.1%), $P=0.005$; data not shown). Finally, among non-diabetics lumican expression was higher in plaques from symptomatic than asymptomatic patients (6.4% (3.8-10.1%) versus 4.7% (1.9-3.8%), $P=0.028$; Figure 2F), while there was no significant difference in the fibromodulin content between symptomatic and asymptomatic plaques in this patient category (Figure 2E).

Association between plaque fibromodulin expression and post-operative cerebrovascular events

Since fibromodulin was increased in plaques from symptomatic patients, we then moved on to test whether fibromodulin expression would be associated with post-operative events. After a mean follow-up time of 37.1 ± 16.3 months, 21% of the patients in our cohort had suffered from one or multiple postoperative CV events. Fibromodulin expression was divided into tertiles, with the highest expression found in the third tertile. Patients with a plaque content of fibromodulin in the second and third tertiles showed an increased incidence of cerebrovascular events – non-fatal (non-hemorrhagic) stroke, TIA or *amaurosis fugax* – as showed by Kaplan–Meier curves of event-free survival ($P = 0.032$ using Log Rank Chi-square test; Figure 3). The higher risk for cerebrovascular events remained significant when controlling for age, gender, diabetes, hypertension, coronary heart disease, smoking and statin use in a Cox Proportional Hazard model

(hazard ratio 4.8, 95% C.I. 1.03-22.37). We found no association between fibromodulin or lumican expression and the occurrence of post-operative AMI, cardiac or vascular interventions (i.e. carotid surgery/stenting, CABG, PCI) or CV (i.e. non-cerebrovascular) death.

DISCUSSION

In the present study fibromodulin content is shown to be higher in plaques from symptomatic patients. Though lumican expression differed only weakly in plaques from symptomatic and asymptomatic patients – and only in patients without diabetes – associations were found between lumican expression and the presence of several features often linked to an unstable plaque phenotype, such as pro-inflammatory cytokines, lipids and macrophages. The correlation between lumican expression and the presence of macrophages is especially noteworthy in light of a previous report showing lumican to directly bind to macrophages and facilitate their migration, suggesting a role for low-sulfate lumican in attracting macrophages to sites of inflammation.²⁶

Fibromodulin expression correlates positively with the expression of the pro-inflammatory cytokines MIP-1 β and s-CD40L, as well as with the expression of VEGF. Plaques with a high expression of fibromodulin also exhibited a higher degree of apoptosis and intraplaque hemorrhage and less smooth muscle α -actin expressing SMCs. Taken together with our previous findings of reduced plaque development in ApoE/fibromodulin-deficient mice¹⁶ the present observations support the notion that fibromodulin influences the development of atherosclerosis, and even suggest a relation with the rupture-prone phenotype of human plaques. ApoE/fibromodulin-deficient mice were also characterized by a reduced retention of lipids in the vascular ECM, and macrophages grown on an ECM lacking fibromodulin had a reduced capacity to take up oxidized LDL, suggesting a role for fibromodulin in vascular retention of LDL.¹⁶ In accordance with these observations we found a significant association between fibromodulin expression and lipid content in human plaques.

We found an inverse association between the plaque contents of fibromodulin and IL-10 in human plaques. However, these data need to be interpreted with some caution because many plaques contained very low or undetectable levels of IL-10. The observation that increased fibromodulin expression was associated with increased expression of some pro-inflammatory cytokines and decreased expression of IL-10 suggest that fibromodulin may have pro-inflammatory properties. In line with this concept we previously observed that macrophages grown on an ECM lacking fibromodulin demonstrate an increased secretion of IL-10¹⁶, also suggesting that fibromodulin may sequester or have an inhibitory effect on the expression of this anti-inflammatory cytokine.

Fibromodulin and lumican expression in human arteries has previously been described by Talusan et al. Deposition of lumican was reported to be enhanced in the intima of the atherosclerosis-prone internal carotid artery compared to the more resistant internal thoracic artery, while fibromodulin expression was similar at both locations.¹⁷ In addition, lumican has been shown to be expressed in intimal vascular SMCs in human coronary atherosclerosis¹⁸ and, as compared to arteries from healthy subjects, overexpressed in arteries from patients with coronary artery dis-

ease,¹⁹ in femoral arteries with atherosclerotic plaques from patients with peripheral occlusive arterial disease²⁰ and in aortic valves from patients with degenerative aortic stenosis²¹.

During collagen fibrillogenesis fibromodulin competes with lumican for collagen binding and is thought to replace collagen-bound lumican as fibril growth progresses.²⁷ Postnatal expression peaks suggest that lumican is mainly active during early fibrillogenesis whereas fibromodulin – though active throughout the process – is especially significant during the later stages.¹¹ Continuous remodeling takes place in atherosclerotic plaques indicating that the processes of collagen breakdown and synthesis occur in parallel. Areas of remodeling thus contain collagen fibrils in different stages of maturation, both early (lumican-driven) and late (fibromodulin-driven). This is also consistent with our observation of fibromodulin and lumican expression in similar plaque regions (in consecutive tissue sections). The main focus on the abilities of fibromodulin and lumican to interact with collagen has so far been on binding sites for collagen types I and II.^{11, 27-30} Thus, not much is yet known about a role for fibromodulin and lumican in synthesis of collagen type III *in vivo*.

Regarding the lack of correlation between fibromodulin and fibrillar structures stained by Masson's trichrome, we made a similar observation in a previous publication on fibromodulin-null mice on an ApoE-null background; there was no difference in the extent of Masson's trichrome staining in ApoE/Fmod-null mice compared to ApoE-null mice.¹⁶ Fibromodulin expression thus does not appear to be associated with the overall ECM content of atherosclerotic lesions, but do correlate specifically with collagen type I immunoreactivity. The notion that lumican detaches from maturing collagen fibrils in favor of fibromodulin during fibrillogenesis,²⁷ may account for the lack of correlation between lumican and mature collagen type I observed in this study.

It is not yet known how long fibromodulin remains attached to the mature collagen fiber after the end of fibrillogenesis. As we found a correlation between fibromodulin and the presence of symptoms we speculate that the effects exerted on plaque phenotype by fibromodulin may – at least partially – be due to the fact that fibromodulin remains bound to the mature collagen fiber for an extended period of time after fibrillogenesis. The different timing for their involvement in collagen formation may also be one explanation for our observation that both fibromodulin and lumican correlate with several – but not quite the same – features of an unstable plaque phenotype. Another possibility is that fibromodulin and lumican have other binding partners, in addition to immature collagen fibrils, and thus an additional mode of influencing plaque phenotype even when not involved in collagen fibrillogenesis. For example, Tillgren et al reported binding between the fibromodulin tyrosin sulfate domain and MMP13.³¹ In the present study, expression of both fibromodulin and lumican correlated positively with MMP1 and -9 as well as with TIMP1 plaque content, leading us to speculate that a direct binding to these enzymes may also take place. Another prospect may be through regulation of angiogenesis; a process in which fibromodulin and lumican appear to have opposite roles. Angiogenesis *in vitro* as well as *in vivo* is promoted by fibromodulin, whereas an inhibitory effect has been reported for lumican.³²⁻³⁶

The connection between diabetes and CV disease is well-documented.³⁷⁻³⁹ Metabolic abnormalities such as chronic hyperglycemia, dyslipidemia and insulin resistance render arteries more susceptible to atherosclerosis.³⁹ In the present study the highest fibromodulin expression was found in symptomatic plaques from patients with diabetes. Fibromodulin levels were also generally

higher in plaques from patients with diabetes than in plaques from non-diabetics. This may be a reflection of an impaired remodeling process in diabetic plaques. An altered ECM remodeling response has been suggested previously in the presence of diabetes. For example, Edsfeldt et al recently reported reduced levels of ECM proteins, elastin, collagen, PDGF and MMP-2 in carotid plaques from diabetic, compared to non-diabetic, patients.²⁵ Furthermore, following MI in diabetic mice delayed collagen accumulation along with higher MMP activity resulted in a disorganized collagen scaffold.⁴⁰

The fact that the association between the expression of fibromodulin and the occurrence of cerebrovascular symptoms also was found during the follow-up of patients can be interpreted as a sign of instability of fibromodulin-rich plaques both in the short and the long term. The finding of an almost 5-time higher risk of suffering of cerebrovascular events could be confirmed after correction for known pro-atherosclerotic conditions suggesting that the quantification of the plaque content of fibromodulin could help in identifying patients at risk of post-operative atherosclerosis-related events. More aggressive primary and secondary prevention measures may be of benefit to such patients.

Taking into account the association between fibromodulin expression and the presence of lipids, pro-inflammatory cytokines and apoptosis, one possible reason for the increased risk of post-operative cerebrovascular events is that the inflammation in the plaque also may extend to a generally inflamed endothelium, as well as the local media or adventitia surrounding the plaque. It is feasible that the inflamed arterial wall continues to affect the local and downstream vascular environment, which may in turn promote the development of further plaques, their growth or destabilization.

Another, perhaps even more likely potential explanation, is that the higher expression of fibromodulin and, indirectly, remodeling gives an indication of the overall inflammatory profile of the patients. A stronger inflammatory presence in these patients might affect both the plaque removed by CEA and as well as other existing plaques; plaques that may exhibit a similar phenotype with a high degree of inflammation and fibromodulin expression/remodeling and are thus also prone to cause cerebrovascular symptoms.

There are some limitations of the present study that should be considered. Most importantly, the associations observed in this study do not provide any proof of causality – the variability of expression of fibromodulin in the current study may be the result more than the trigger of the occurrence of pre-operative symptoms. However, there was not a significant correlation between the time between symptoms and the operation and the levels of the SLRPs measured here. Nonetheless, the association between plaque fibromodulin and lumican and plaque lipids may not reflect an ability of fibromodulin to retain plasma lipids as suggested by the studies performed in ApoE/fibromodulin-deficient mice¹⁶ but rather that fibromodulin is up-regulated in more advanced, lipid-rich plaques. Another limitation is that registry-based follow-up of events will not account for clinically silent events.

CONCLUSIONS

The present findings of increased expression of fibromodulin in symptomatic human carotid plaques taken together with our previous experimental studies of decreased vascular retention of lipids and reduced plaque development in ApoE/fibromodulin deficient mice lacking fibromodulin¹⁶ suggest that fibromodulin may be directly involved in the development of atherosclerotic plaques, particularly in diabetic patients. The differing correlations between fibromodulin and lumican with symptoms and plaque components points toward the existence of unique roles in atherogenesis for the two proteoglycans, despite close homology in structure and function. The finding that fibromodulin levels in the plaque predict future cerebrovascular events further emphasizes the possible importance of pathways involving SLRPs in diagnosis and treatment of atherosclerosis. Further studies are needed to characterize the mechanisms through which fibromodulin and lumican may influence plaque development.

ACKNOWLEDGEMENTS

The authors wish to thank Lena Sundius, Anki Knutsson, Sabrina Hsiung, Mihaela Nitulescu, Ana Persson, Fong To and Dr. Pontus Dunér, for expert technical assistance and Dr. Sebastian Kalamajski and Prof. Åka Oldberg for valuable discussion.

FUNDING

This work was supported by the Swedish Heart-Lung Foundation [grant numbers 20120196, 20141785]; the Swedish Research Council [grant number VR-K2013-64X-22299-01-3]; Swedish Foundation for Strategic Research [grant number SSF-RBa08-0075]; Alfred Österlund Foundation; the Kock Foundation; the Crafoord Foundation; Thelma Zoéga's Foundation; the Royal Physiographic Society in Lund; Marianne and Marcus Wallenberg Foundation [grant number MMW2010.0040]; Swedish Medical Society [grant number SLS 249041]; Lundström's foundations [n/a]; and Skåne university hospital grants [grant number 1400741].

CONFLICT OF INTEREST

None declared.

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FIGURES

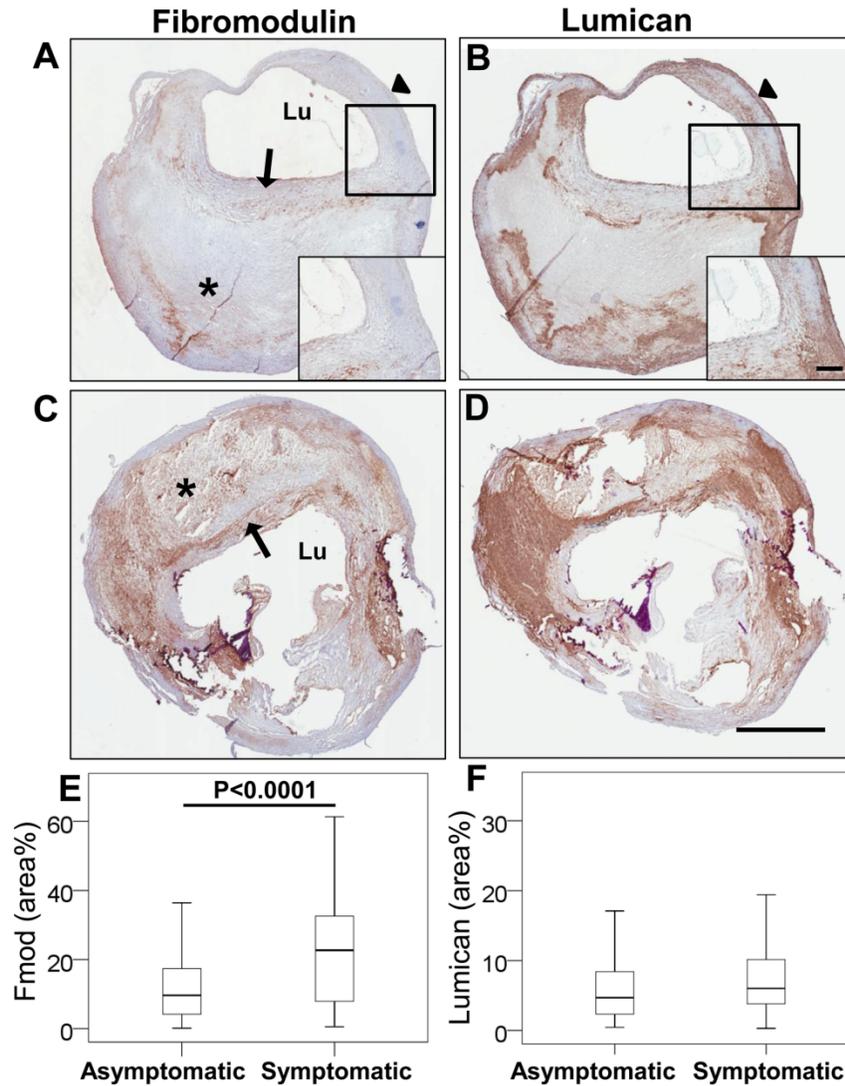


Figure 1. Representative stainings of fibromodulin and lumican in asymptomatic (A and B, respectively) and symptomatic (C and D, respectively) plaques. Magnified areas are denoted by black squares. The vessel lumen is denoted by *Lu*, the cap region by arrows, the core by asterisks, and the plaque/media interface by arrowheads. Scale bars are 2 mm and 400 μ m, respectively. Box plots show fibromodulin (Fmod) and lumican content in plaques from patients with or without symptoms (E and F, respectively). Error bars show interquartile range.

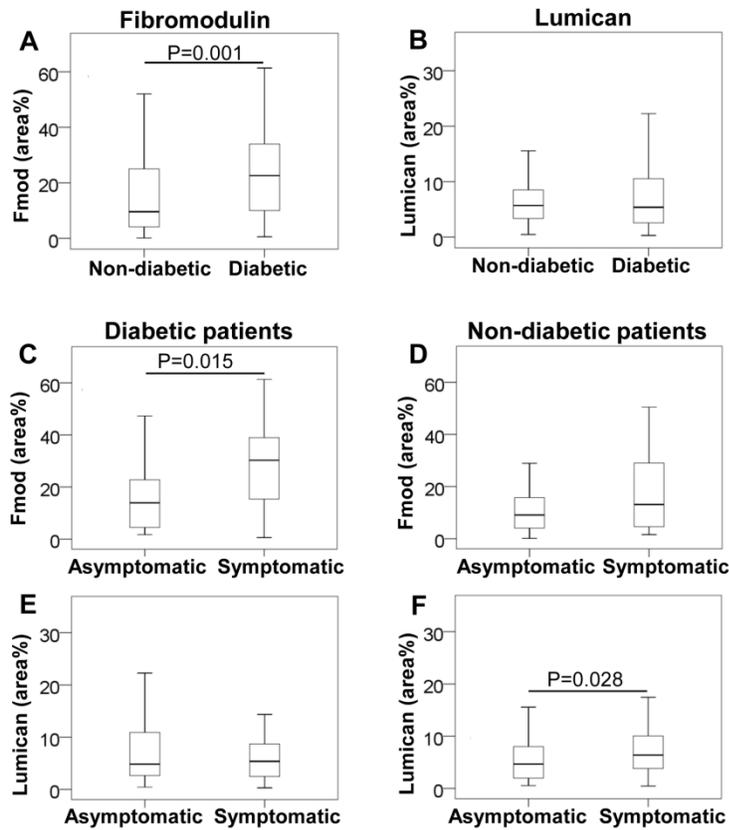


Figure 2. Box plots showing fibromodulin (Fmod) and lumican content in plaques from patients with or without diabetes (A and B, respectively) and in plaques from diabetic (C and E, respectively) and non-diabetic patients (D and F, respectively) grouped based on the occurrence of pre-operative symptoms. N=153 plaques. Error bars show interquartile range.

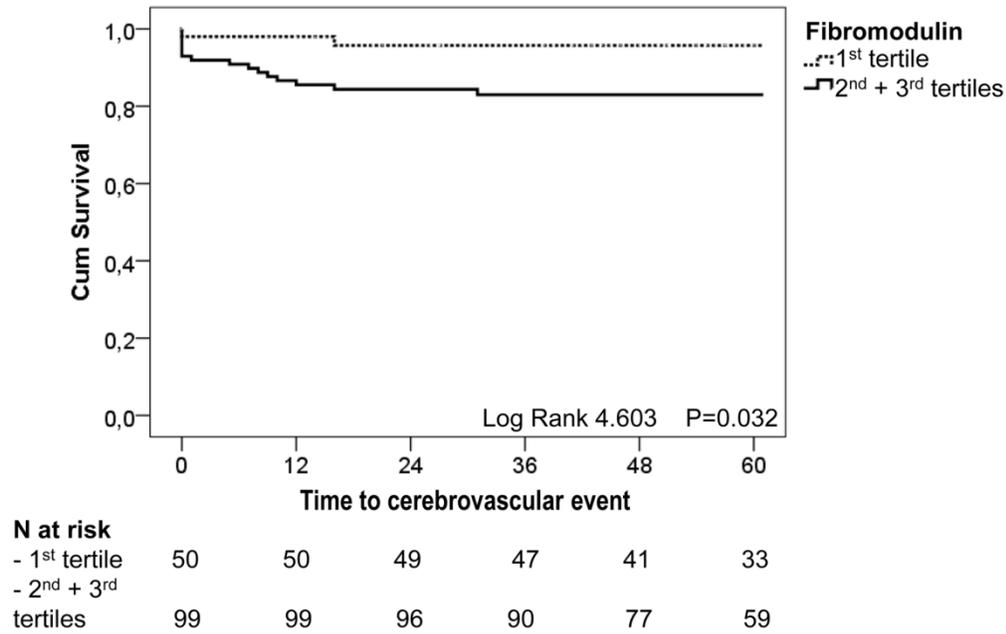


Figure 3. Kaplan-Meier survival analysis with patients grouped based on tertiles of plaque content of fibromodulin (1st versus 2nd+3rd tertiles) and time to cerebrovascular events expressed in months. N=149 patients and numbers (N) at risk in the two groups at different time points are indicated below the figure.

TABLES

Table 1. Spearman correlations between fibromodulin (% area), lumican (% area) and other plaque components analyzed by histology (% area). Ns = not significant.

Histology	Fibromodulin		Lumican	
	<i>r</i>	P	<i>r</i>	P
Elastin	0.065	ns	0.212	0.013
Masson tri- chrome	0.126	ns	0.080	ns
Oil Red O	0.173	0.036	0.206	0.012
Calcium	-0.80	ns	-0.79	ns
CD68	0.035	ns	0.229	0.005
Collagen type I	0.167	0.039	-0.008	ns
Collagen type III	-0.064	ns	0.002	ns
Glycophorin A	0.356	<0.001	0.355	<0.001
Smooth muscle α -actin	-0.196	0.018	-0.148	ns
Fibromodulin			0.287	<0.001
Lumican	0.287	<0.001		

Table 2. Spearman correlations between fibromodulin (% area), lumican (% area) and cytokines (pg/g plaque wet weight), measured by Luminex assays. Ns = not significant.

Cytokine	Fibromodulin		Lumican	
	<i>r</i>	P	<i>r</i>	P
IL-10	-0.281	0.002	0.028	ns
MIP-1 β	0.189	0.037	0.088	ns
s-CD40L	0.188	0.040	-0.052	ns
PDGF	-0.075	ns	0.024	ns
RANTES	0.025	ns	0.190	0.037
TNF- α	0.127	ns	-0.042	ns
VEGF	0.208	0.025	-0.177	ns

ONLINE SUPPLEMENT

Expression of fibromodulin in carotid atherosclerotic plaques is associated with diabetes and cerebrovascular events

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SUPPLEMENTARY MATERIALS AND METHODS

Patients

All patients underwent a standardized ultrasound examination of the carotid arteries the day before surgery and were clinically assessed preoperatively by an independent accredited neurologist. The indications to surgery were as described previously¹; briefly, carotid artery stenosis associated with ipsilateral symptoms and a stenosis degree > 70% or, in patients without cerebrovascular symptoms, a stenosis degree > 80 %. The stenosis degree was assessed with ultrasound based on flow velocities as previously validated.² Patients were considered to have asymptomatic disease if they had had no *amaurosis fugax*, transient ischemic attacks (TIAs) or strokes in the 6 months prior to surgery.

Patients were defined as suffering of diabetes if diagnosed with diabetes mellitus at the time of plaque removal or had been diagnosed previously, or were undergoing anti-diabetic treatment. The majority of patients with diabetes had type 2 diabetes (n=48/50).

Postoperative events

The Swedish national inpatient health register was analyzed in order to identify postoperative CV events, with corresponding International Classification of Diseases, Tenth Revision (ICD-10) codes G45, G46, I20 to I25, I60 to I69 and I97 from January 1998 to December 2010.

Definition of outcomes All events occurring in the postoperative period were analyzed singularly. Events occurring in the first 24 hours postoperatively were considered as procedure-related and excluded from the follow-up analysis. Furthermore, all cerebrovascular events were analyzed in combination. The composite variable CV event was defined as including any death of CV origin, non-fatal stroke (non-hemorrhagic), non-fatal acute myocardial infarction (AMI), and any TIA or *amaurosis fugax*. Any arterial cardiac or vascular intervention that had not already been planned at the time of inclusion such as carotid surgery or stenting, coronary artery bypass grafting (CABG), percutaneous coronary artery intervention (PCI) was also registered and analyzed singularly.

Patients suffering of more than one episode of the same event (i.e. patients with for example multiple strokes or AMIs) were classified as suffering of multiple events. In these cases only the first chronological event was taken into account in the survival analysis. A CABG or a PCI performed during the first 2 weeks after an AMI as well as a later surgical or endovascular intervention for a symptomatic contralateral carotid artery stenosis/ipsilateral restenosis were considered as consequence of the correlated cardiac/neurologic ischemia and not recorded as supplementary events.

Sample Preparation

Carotid plaques were snap-frozen immediately after surgical removal. One-millimeter-thick fragments from the most stenotic region of the plaque were used for histological analyses and embedded in optimal cutting medium (Sakura Finetek Europe BV, Japan) and cryosectioned (8 μ m). The rest of the plaques was homogenized as described previously³, with the addition of 100 mM EDTA/EGTA to homogenates.

Immunohistochemistry and Histology

For histological characterization of the plaque components, sections were fixed with Histochoice (Amresco, Solon, OH, USA). A Hematoxylin & Eosin (H&E) stain was performed using the Mayer's Hematoxylin (Histolab, Gothenburg, Sweden). Lipid (Oil Red O), Masson's trichrome and elastin stains were performed as previously described.^{4,5}

Carotid plaque sections were immunohistochemically stained using primary antibodies against fibromodulin (kindly provided by D. Heinegård, Lund University, Sweden) at 1:1000 dilution, lumican at 1:400 dilution (ab168348; Abcam, Cambridge, UK), Glycophorin A at 1:400 dilution (M0819, Dako Sweden, Stockholm, Sweden) and collagen I and III at 1:2000 and 1:4000 dilution (ab6308 and ab6310 respectively; Abcam, Cambridge, UK). Prior to incubation with the fibromodulin, lumican and Glycophorin A antibodies sections were permeabilized with 0.5% Triton X-100 for 5 minutes, and prior to incubation with the collagen antibodies sections were pre-treated with sodium citrate antigen retrieval buffer (pH 6) at 100 °C for 15 minutes. Blocking was performed in 10% goat- or rabbit-serum (depending on secondary antibody host species), while primary antibodies were diluted in 1 % goat- or rabbit-serum. The secondary antibodies used were biotinylated goat anti-rabbit IgG (Vector BA-1000, Vector Laboratories Inc, Burlingame, CA, USA – fibromodulin and lumican stains) or biotinylated rabbit F(ab')₂ anti-mouse IgG

(E0413, Dako Sweden, Stockholm, Sweden for the Glycophorin A stain, and ab98668, Abcam, Cambridge, UK for the collagen stains) – dilutions ranging from 1:200 to 1:500 in 1 % goat- or rabbit serum.

Isotype control antibodies were used in concentrations corresponding to that of each primary antibody (ab27478 and ab81032 from rabbit and mouse, respectively; Abcam, Cambridge, UK). In the case of the fibromodulin antibody, as a negative control the primary antibody was omitted during staining and the antibody has also been tested on fibromodulin-null carotid artery lesions⁶ where it produced no positive immunoreactivity.

In addition, vascular smooth muscle cells (SMCs; marker smooth muscle α -actin) and macrophages (CD68) were immunohistochemically stained as previously described.^{4, 7} Positive immunoreactivity was visualized using 3,3'-diaminobenzidine (DAB; Vector Laboratories Inc, Burlingame, CA, USA). Sections were counterstained with Mayer's Hematoxylin (Histolab, Gothenburg, Sweden). Stained sections were scanned and digitalized using Aperio ScanScope digital slide scanner (Aperio Technologies, Inc, Vista, CA) and immunoreactivity was quantified using the imaging software program BioPix iQ version 2.3.1 (Biopix Ab, Gothenburg, Sweden). Residual media was not included during image analysis.

MMP and TIMP Assessment

Analysis of (MMPs) and (TIMPs) were performed. Different matrix metalloproteinases (MMPs; 1-3, 7, 9 and 12) were analyzed in human plaque homogenate supernatants (25 μ L) using Mesoscale human MMP ultra sensitive kit (Mesoscale, Gaithersburg, MD, USA), according to the manufacturer's instructions. Tissue inhibitors of MMPs (TIMPs; 1 and 2) were analyzed in human plaque homogenate supernatants using MILLIPLEX MAP Human TIMP Magnetic Bead Panel (Milliplex, MA, USA), according to the manufacturer's instructions.⁸

Cytokine Assessment

Aliquots of plaque homogenate (50 μ L) were centrifuged at 13000 g for 10 minutes. The supernatant (25 μ L) was removed and used for measuring interleukin (IL), macrophage inflammatory protein 1 β (MIP-1 β), regulated on activation, RANTES, soluble CD40 Ligand (sCD40L), tumor necrosis factor- α (TNF- α), platelet-derived growth factor (PDGF) and vascular endothelial

growth factor (VEGF). The procedure was performed according to the manufacturer's instructions (Human Cytokine/Chemokine Immunoassay, Millipore Corporation, MA, USA) and analyzed with Luminex 100 IS 2.3 (Austin, TX).

SUPPLEMENTARY TABLES

Table I. Clinical characteristics of patients (n=150) depending on occurrence of symptoms. Values shown are median with interquartile range. NS = not significant.

	Asymptomatic (n=72)	Symptomatic (n=78)	Sig.
Sex (% male)	69	68	NS*
Age (years)	68 (64-72)	73 (66-78)	P<0.0001
Body mass index	26.4 (24-29)	25.9 (24.2-28.6)	NS
Smoking, currently or in the past (%)	79	82	NS
Diabetes (%)	24	56	P=0.008
Hypertension (%)	76	74	NS
Degree of stenosis (%)	90 (81-95)	90 (75-95)	NS
Statins (%)	90	79	NS
Fasting lipoproteins, (mmol/L)			
Total cholesterol	4.3 (3.5-5.1)	4.3 (3.5-5.0)	NS
LDL cholesterol	2.4 (1.9-3.1)	2.5 (2.0-3.1)	NS
HDL cholesterol	1.1 (0.9-1.3)	1.1 (0.9-1.3)	NS
Triglycerides	1.3 (0.9-1.8)	1.2 (0.8-1.7)	NS

*Not significant

Table II. Clinical characteristics of patients (n=150) depending on the presence of diabetes. Values shown are median with interquartile range. NS = not significant.

	Non-diabetic (n=99)	Diabetic (n=51)	Sig.
Sex (% male)	72	63	NS*
Age (years)	69 (64-74)	70 (67-76)	NS
Body mass index	25.8 (23.6-27.4)	27.6 (25.2-30.7)	P=0.001
Smoking, currently or in the past (%)	81	80	NS
Hypertension (%)	74	78	NS
Degree of stenosis (%)	90 (80-95)	90 (80-95)	NS
Statins (%)	85	84	NS
Fasting lipoproteins, (mmol/L)			
Total cholesterol	4.1 (3.3-4.9)	4.1 (3.3-5.0)	NS
LDL cholesterol	2.5 (2.0-3.2)	2.2 (1.8-3.0)	NS
HDL cholesterol	1.1 (0.9-1.3)	1.0 (0.9-1.3)	NS
Triglycerides	1.2 (0.8-1.6)	1.5 (1.1-1.8)	P=0.007
HbA1c (mmol/mol)	40.1 (38.7-43.2)	51.0 (45.8-60.4)	P=0.002

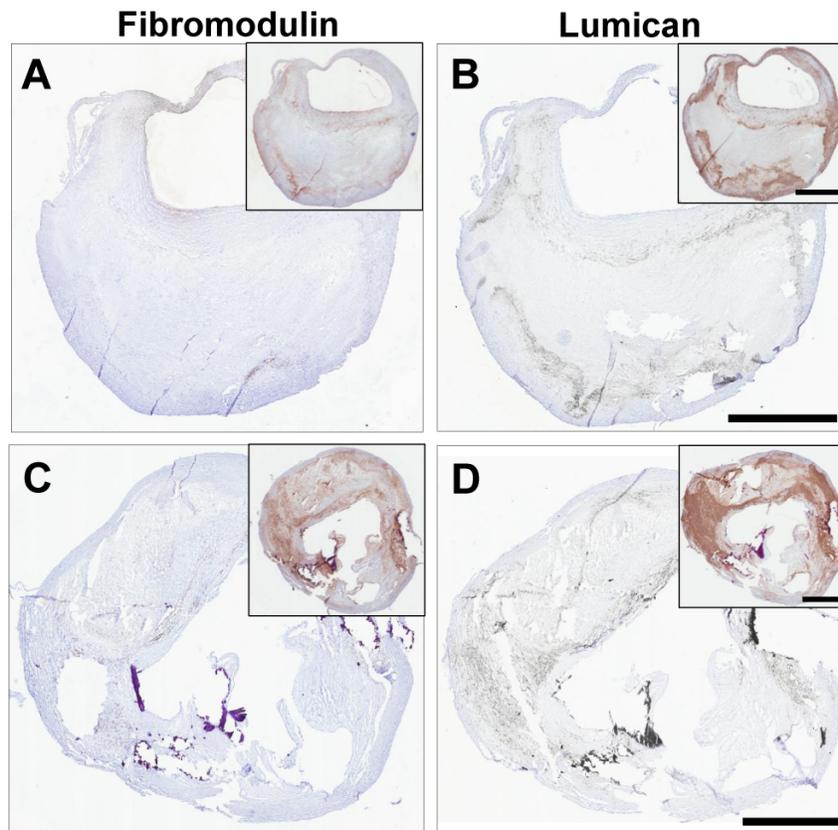
*Not significant

Table 3. Spearman correlations between fibromodulin (% area), lumican (% area) and matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs; pg/g plaque wet weight), measured by Luminex assays. Ns = not significant.

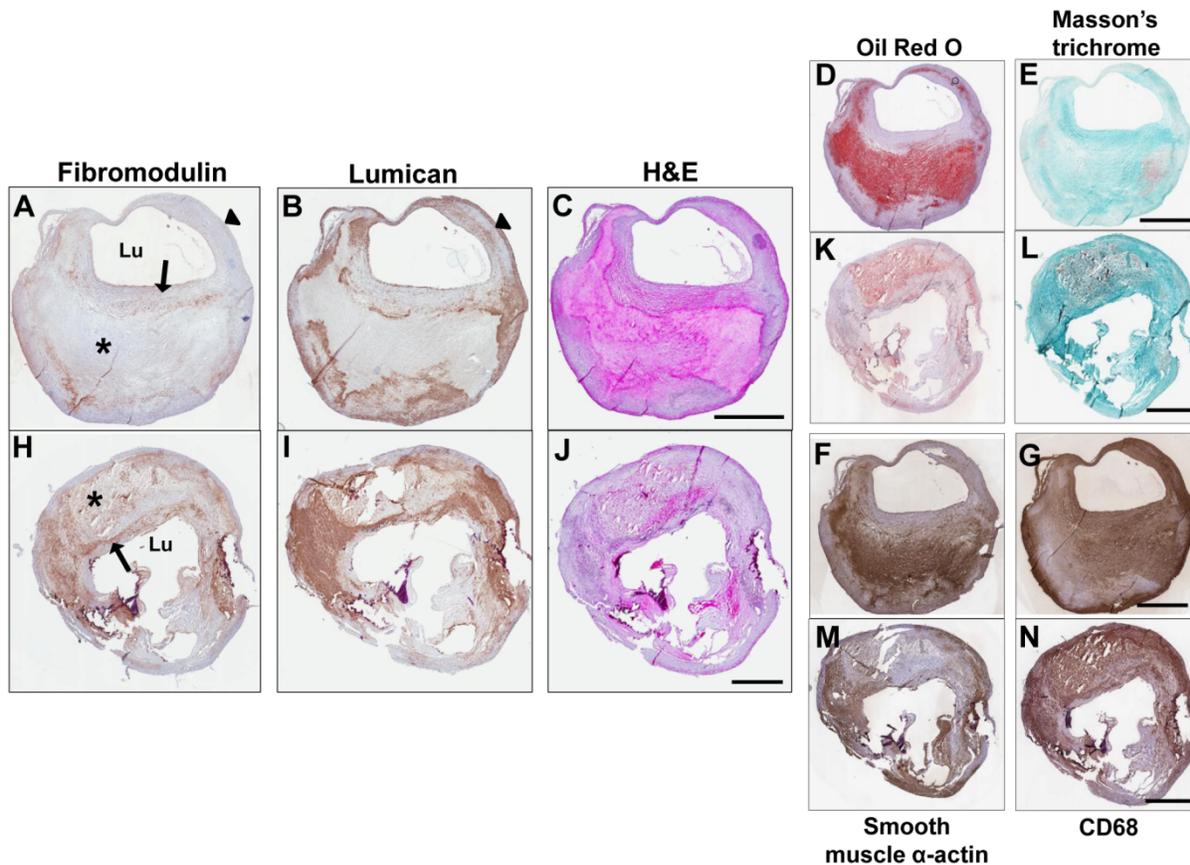
	Fibromodulin		Lumican	
	<i>r</i>	P	<i>r</i>	P

MMP1	0.179	0.037	0.209	0.015
MMP2	-0.062	ns	0.061	ns
MMP3	-0.134	ns	-0.094	ns
MMP7	0.140	ns	0.205	0.023
MMP9	0.290	0.001	0.332	<0.001
MMP12	-0.184	0.042	-0.116	ns
TIMP1	0.245	0.004	0.208	0.015
TIMP2	0.002	ns	-0.027	ns
TIMP3	0.113	ns	0.116	ns

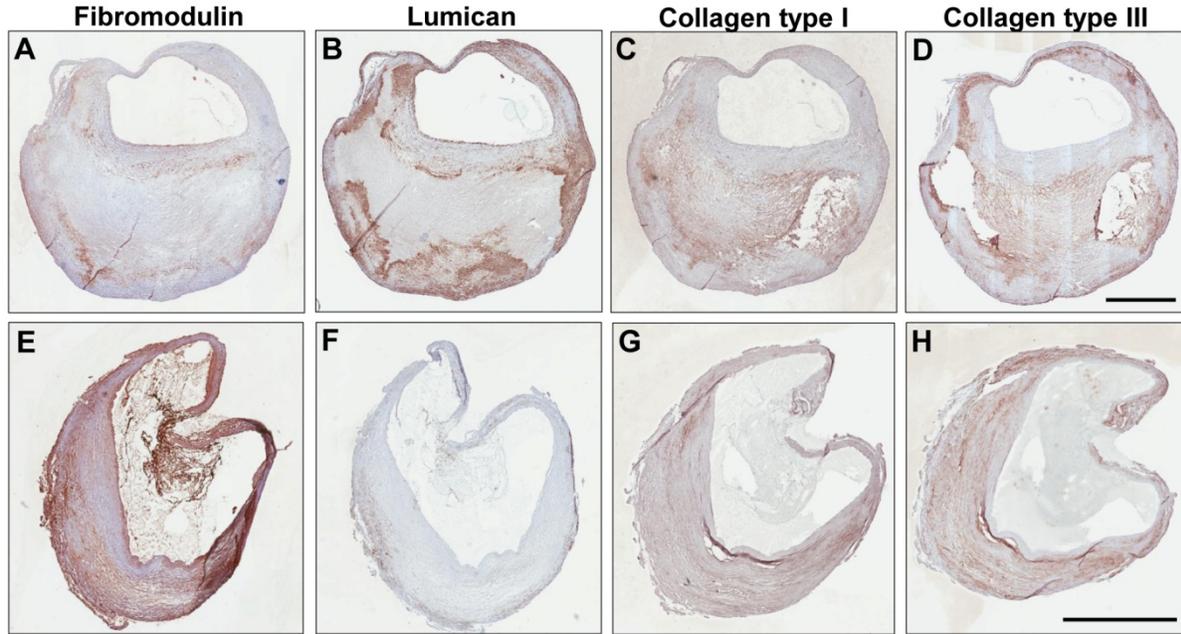
SUPPLEMENTARY FIGURES



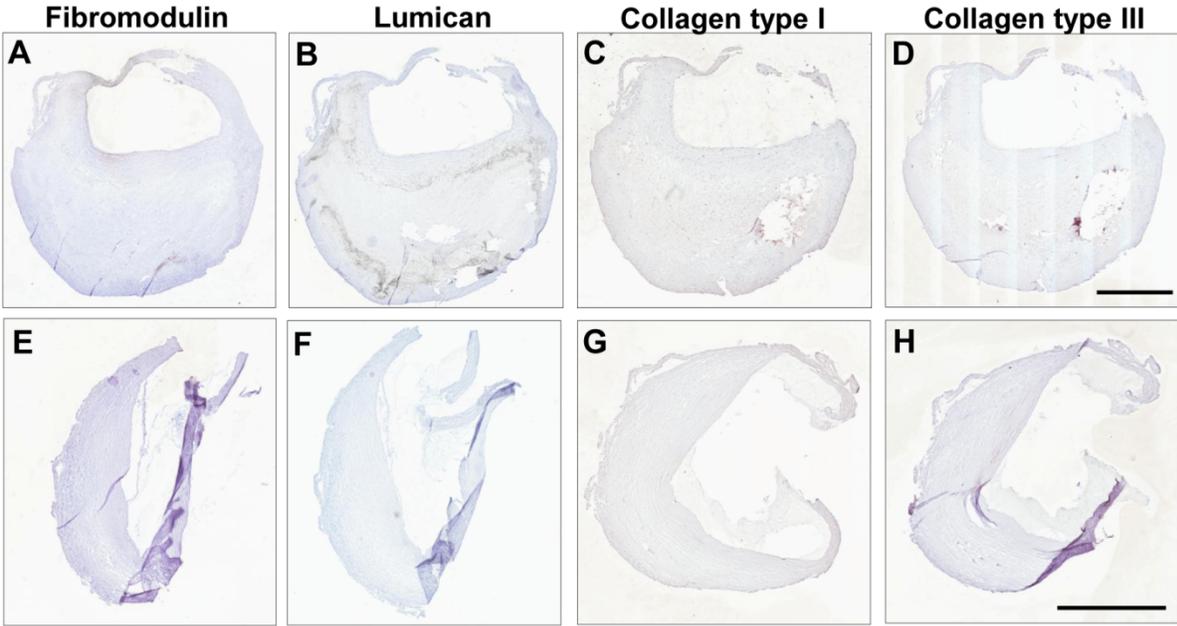
Supplementary Figure I: Negative controls, Fig. 1. Representative images of negative controls used in immunohistochemical stainings of fibromodulin (A and C) and lumican (B and D) shown in Fig. 1. Immunohistochemical stainings from Fig. 1 are shown as insets. Scale bars are 2 mm.



Supplementary Figure II: Histological overview. Representative stainings of fibromodulin (A and H), lumican (B and I), Hematoxylin and Eosin (H&E; C and J), Oil Red O (D and K), Masson's trichrome (E and L), smooth muscle α -actin (F and M) and CD68 (G and N) in plaques from asymptomatic (A-G) and symptomatic (H-N) patients. The vessel lumen is denoted by *Lu*, the cap region by arrows, the core by asterisks, and the plaque/media interface by arrowheads. Scale bars are 2 mm.



Supplementary Figure III: Collagen types I and III. Representative immunohistochemical stainings of fibromodulin (A and E), lumican (B and F), collagen type I (C and G) and collagen type III (D and H) in plaques from asymptomatic (A-D) and symptomatic (E-H) patients. Scale bars are 2 mm.



Supplementary Figure IV: Negative controls, Supplementary Figure III. Representative images of negative controls used in immunohistochemical stainings of fibromodulin (A and E), lumican (B and F), collagen type I (C and G) and collagen type III (D and H) in the atherosclerotic plaques from asymptomatic (A-D) and symptomatic (E-H) patients shown in Supplementary figure III. Scale bars are 2 mm.

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